Molecular Recognition in a Post-translational Modification of Exceptional Specificity

MUTANTS OF THE BIOTINYLATED DOMAIN OF ACETYL-CoA CARBOXYLASE DESTRUCTIVE IN RECOGNITION BY BIOTIN PROTEIN LIGASE*

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We have used localized mutagenesis of the biotin domain of the Escherichia coli biotin carboxyl carrier protein coupled with a genetic selection to identify regions of the domain having a role in interactions with the modifying enzyme, biotin protein ligase. We purified several singly substituted mutant biotin domains that showed reduced biotinylation in vitro and characterized these proteins in vitro. This approach has allowed us to distinguish putative biotin protein ligase interaction mutations from structurally defective proteins. Two mutant proteins with glutamate to lysine substitutions (at residues 119 or 147) behaved as structurally defective ligase interaction mutants. The E119K protein was virtually inactive as a substrate for biotin protein ligase, whereas the E147K protein could be biotinylated, albeit poorly. Neither substitution affected the overall structure of the domain, assayed by disulfide dimer formation and trypsin resistance. Substitutions of the highly conserved glycine residues at positions 133 and 143 or at a key hydrophobic core residue, Val-146, gave structurally unstable proteins.

Biotin is an essential coenzyme that has both metabolic activity only when covalently attached to a class of important metabolic enzymes, the biotin carboxylases and carboxytransferases (1, 2). Biotin is attached via an amide linkage to a specific lysine residue of the cognate protein, and this reaction is catalyzed by biotin ligase (BPL, also known as carboxylase synthetase) in two-step Reaction 1 as follows.

Step 1: Biotin + ATP ⇌ biotinoyl-AMP + PP,

Step 2: Biotinyl-AMP + apoprotein → biotinoyl-protein + AMP

This is a post-translational modification of extraordinary specificity. For example BPL recognizes just one of the >4000 different protein species of Escherichia coli, the biotin carboxyl carrier protein (BCCP), and quantitatively attaches biotin to a specific lysine residue of this protein. BCCP is one of the four protein species that comprise acetyl-CoA carboxylase (EC 6.4.1.2), the enzyme catalyzing the first committed step of fatty acid biosynthesis, the conversion of acetyl-CoA to malonyl-CoA (3). Biotinylation is a relatively rare post-translational modification throughout biology, with between one and five biotinylated protein species found in different organisms (4). The sequences of both the BPLs and biotin acceptor protein domains are highly conserved. Moreover, this conservation applies to ligase-domain interactions, since biotinylation occurs only when enzyme and protein substrate are derived from widely divergent species (4–7).

The three-dimensional structure of the biotinylated (holo) form of the biotin domain of E. coli BCCP has been determined by both NMR and x-ray crystallography (8), giving essentially identical structures. The protein forms a β-barrel structure, with the biotinyl-lysine exposed on a tight β-turn within the conserved Ala-Met-Lys-Met biotinylmation domain of the BCCP biotin domain adopts a fold similar to that of several lipoyl domains (9–11) which undergo an analogous post-translational modification. The lipoyl cofactor is covalently attached to a specific lysine residue within a highly conserved Asp-Lys-Ala motif by lipoyl ligation via an ATP-activated intermediate (12–13). The structure of the unbiotinylated (apo) form of the BCCP biotin domain determined by NMR (14) is very similar to that of the holoenzyme, with both forms of the protein having the same basic fold and some localized small differences. However, we have demonstrated recently that there is a subtle, global alteration in the structure of the domain accompanying biotinylation which can be detected by proteolysis and chemical modification (15). Thus, it appears that these techniques, probably as a consequence of their irreversible nature, are a very sensitive indicator of changes in protein dynamics (16).

The biotin-accepting domain of BCCP undergoes a complex series of protein-protein interactions, since it must interact with BPL to become functional and then with both the carboxylase and carboxyltransferase active sites of the acetyl-CoA carboxylase complex. The precise structural elements within the biotin domain that direct post-translational modification of the specific lysine are unknown. Several studies have shown that the nature of the flanking methionine residues, although not essential for biotinylation, does have an effect on the effi...
ciency of the reaction. Substitution of the Met-Lys-Met sequence with the Asp-Lys-Ala lipoylation motif abolishes biotinylation, and changing either of the Met residues to Lys significantly reduces the extent of modification (17). However, more conservative substitutions of the flanking methionines have little adverse effect on biotinylation (18, 19) but do effect the carboxylation and carboxyl transfer reactions of *Propionibacterium shermanii* transcarboxylase (20). The extent to which truncated forms of biotin carrier proteins are biotylated indicates that a minimum of 35–40 residues on either side of the biotin attachment site is necessary to specify biotinylation (3, 4). It is now evident that further truncation, which abolishes biotinylation, removes residues that contribute to the formation of the hydrophobic core of the folded structure (8). Thus, it is clear that BPL recognizes the Met-Lys-Met motif within the context of a folded protein (21). Therefore, mutant proteins that fold improperly are expected to be poor biotin acceptors. Indeed, amino acid substitutions at one of the several highly conserved glycine residues, or within the conserved Pro-X-X-Gly motif found N-terminal to the biotin attachment site, severely reduce the efficiency of biotinylation of the biotin domain from human propionyl-CoA carboxylase in *E. coli* (19). However, many of these substitutions, especially those of the Gly residues, would be expected to destabilize the structure of the domain and thus the poor biotinylation could well be a secondary consequence of the altered structure rather than a specific defect in recognition by BPL. Such structural alterations cannot be readily detected in vivo.

In order to identify recognition determinants within the folded domain, we have addressed the problem of structural alteration introduced by amino acid substitution. We used localized mutagenesis of the C-terminal 87 residues of the *E. coli* BCCP biotin domain (BCCP87) coupled with a genetic selection to identify regions of the domain that are candidates for a role in biotin domain-BPL interactions. We purified several of these mutant biotin domain proteins that showed reduced biotinylation in vivo and have characterized the defect in vitro. This approach has allowed us to distinguish putative BPL interaction mutations from structurally defective proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—Generalized transduction with P1vir and bacteriophage plates containing 5–200 nM biotin, respectively were annealed as described by Kalderon et al. (33) and subcloned to the annealing buffer to produce gapped heteroduplexes with 0.3-kb single-stranded loops. These heteroduplexes were subjected to local mutagenesis by treatment with 1.0 mM methoxylamine hydrochloride for 3–60 min essentially as described (34, 35). After removal of methoxylamine and ethanol precipitation, the treated DNA was transformed directly into TM21, with selection for ampicillin resistance on rich broth plates. The resulting colonies were then screened for production of the ACP-BCCP fusion by scoring growth on Macokey lactose medium. The lactose induces high level expression of the gene encoding the fusion protein. If the fusion protein is produced and stable, induction will result in growth inhibition due to titration of biotin ligase and the intrinsic toxicity of ACP overproduction (25). Strains sensitive to lactose were then screened for derepression of biotin ligase expression (see below), and the remaining candidates were assayed for production of the full-length fusion protein and biotinylation by radioactive labeling in vivo (see below).

**Derepression of bio-lacZ Fusion Strains**—Strains of interest were scored by a radial streak assay in which single colonies were suspended in 0.5 ml of medium E and then streaked outward from the center of minimal E plates containing 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal; 80 μg/ml) and lacking biotin. A filter paper disc in the center of the plate was then spotted with 20 μl of 500 μM biotin. After overnight incubation at 37 °C, bio-lacZ derepression resulted in a sharp blue/white interface near the outer edge of each growth streak. The distance between this interface and the biotin-saturated disc was taken as an indicator of biotin consumption. For quantitative assessment of derepression, the β-galactosidase activity of liquid cultures was measured as described by Miller (22), with cells disrupted with SDS and chloroform.

**Protein Expression, Purification, and Analysis**—Growth experiments requiring minimal media were carried out in medium E (36) supplemented with 0.4% glucose, 1 μM thiamine, 0.1% vitamin-free casamino acids, and the indicated concentrations of biotin and β-alanine. BCCP encoding BCCP or ACP in the fusion protein, these media contained either 75–200 mM [3H]biotin (1 μCi/ml) and 10 μM β-alanine or 2.5–5 [3H]β-alanine (3–25 μCi/ml) and 100–200 mM biotin, respectively. After growth overnight in 0.2 ml of [3H]-containing media, cells were subcultured into 1.0 ml of fresh [3H] media, and fusion protein production was induced by the addition of isopropylthigalactoside (IPTG) to 1 mM. Whole cell lysates were prepared for SDS-PAGE essentially as described by Chapman-Smith et al. (32) except that the reductant used was 10 mM dithiothreitol. Sample loading was normalized by optical density measurements prior to electrophoresis. Gels were fixed in 10% acetic acid, 10% methanol, treated with Enlightening (NEN Life Science Products) for 30 min, dried under vacuum, and exposed to prefetched x-ray film at 70 °C.

Isolated wild type and mutant biotin domain peptides were expressed from derivatives of pTM53 in strain BL21(ADE3) and purified essentially as described previously (32). Additional purification by gel filtration chromatography using Superdex 75 (Amersham Pharmacia Biotech) and reduction of disulfide-bonded dimers were carried out as described in Chapman-Smith et al. (15). Trypsin digestion, high performance liquid chromatography analysis, and peptide quantitation were carried out as described previously (15, 32). Other protein methods and analyses were as described previously (15, 32) except that trypsin digestions were done in the presence of 1 mM DTT. Preliminary experiments showed that trypsin retained activity in the presence of 1 mM DTT using apoBCCP as a substrate, whereas 10 mM DTT inhibited the activity 3–4-fold (data not shown).

**Biotinylation Assays**—Biotinylation assay activity was measured by following incorporation of [3H]biotin into acid-precipitable material over time, with either wild type apoBCCP or the mutant apoproteins as the biotin acceptor. Unless otherwise stated, the assays contained 40 mM Tris-HCl, pH 8.0, 3 mM ATP, 5.5 mM MgCl₂, 5 μM biotin, 5 μM of

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3 J. Cronan, unpublished results.
\[^{[3}H\]biotin (specific activity 35–44 Ci/mmol), 100 mM KCl, 1.4 mM \(\beta\)-mercaptoethanol, 0.1 mg/ml bovine serum albumin carrier protein, and the indicated concentrations of apoprotein in a final volume of 100 \(\mu\)l. The reaction was initiated by addition of purified \(E.\ coli\) biotin ligase (BirA; the gift of Dr. Dorothy Beckett) to a final concentration of 12.5 \(\mu\)M and incubated at 37 °C for up to 30 min, during which time the reaction was linear at saturating substrate concentrations. Aliquots taken at various time intervals were spotted onto dry 2 \(\times\) 2-cm squares of Whatman 3MM paper to which 100 \(\mu\)l of 5 mM biotin and 100 \(\mu\)l of 10% trichloroacetic acid had previously been applied. The pretreatment with trichloroacetic acid was found to be necessary to prevent the reaction continuing on the filter. After air-drying, the filters were washed batchwise twice in ice-cold 10% trichloroacetic acid and once in ethanol, dried, and the acid-insoluble radioactivity measured. The optimal pH for activity was determined in assays over a pH range 4.5–11.0, with the following buffer systems at 40 mM: sodium acetate, pH 4.5–5.5; sodium phosphate, pH 6.0–7.5; sodium MOPS, pH 6.0–8.0; Tris-HCl, pH 7.0–9.5, and sodium CAPS, pH 9.0–11.0. For analysis of kinetic experiments, the maximum amount of added \(^{[3}H\)biotin that could be fixed by the enzyme was determined from a reaction with 0.25 \(\mu\)M enzyme (since \(^{[3}H\)biotin had a 3-fold lower counting efficiency on the filter paper when not bound to protein). In some experiments, to obtain sufficiently high levels of radioactivity for accurate detection, it was necessary to allow the reaction to continue until greater than 10% of the limiting substrate had been utilized. In this case the data were transformed for altering substrate concentration by the method of Lee and Wilson (37) and plotted as transformed values \(s^*\) and \(v^*\). Values for \(K_m\) and \(V_{max}\) were determined by fitting a plot of substrate concentration against rate directly to the Michaelis-Menten equation using GraphPad Prism for MacIntosh (GraphPad Software Inc, San Diego, CA). In cases where a reasonable estimate of \(V_{max}\) could not be obtained due to practical limitations to the substrate concentration range, constants were determined by linear regression with data in double-reciprocal form using GraphPad Prism.

**RESULTS**

**Expression of ACP-BCCP Fusions**—In order to isolate mutant biotin domains deficient in biotinylation in vivo, we constructed protein fusions to the BCCP biotin domain that could be detected using a straightforward assay that did not depend on biotinylation (Fig. 1A). Acyl carrier protein (ACP) from \(E.\ coli\) was chosen as the N-terminal reporter group for the fusions because it is small (77 amino acids), stable, and can be specifically labeled (through its covalently linked 4'-phosphopantetheine prosthetic group) with \(^{[3}H\)β-alanine (38). The ACP-BCCP fusion plasmid pTM4D encoded the 70 N-terminal residues of ACP followed by a short artificial linker and then the C-terminal 87 residues of BCCP (BCCP87). BCCP87 was chosen as the fusion sequence because fusion proteins that encoded only the last 55 or 67 residues of BCCP were not chosen as the fusion sequence because it is small (77 amino acids), stable, and can be specifically labeled (through its covalently linked 4'-phosphopantetheine prosthetic group) with \(^{[3}H\)β-alanine (38). The ACP-BCCP fusion plasmid pTM4D encoded the 70 N-terminal residues of ACP followed by a short artificial linker and then the C-terminal 87 residues of BCCP (BCCP87). BCCP87 was chosen as the fusion sequence because fusion proteins that encoded only the last 55 or 67 residues of BCCP were not biotinylated in vivo (39). The lac\(^{+}\) host strains TM5 and TM21 required both biotin and \(\beta\)-alanine for growth which allowed efficient labeling and detection of the expressed fusion proteins. When expressed in the presence of \(^{[3}H\)biotin or \(^{[3}H\)β-alanine and analyzed by SDS-PAGE (“Experimental Procedures”), the ACP-BCCP fusion was efficiently and specifically labeled with both radioactive compounds (Fig. 1B), indicating that both components were properly recognized by their cognate modification enzymes (BPL and holo-ACP synthase). Moreover, the native forms of both modified proteins were also present thus providing internal standards for the labeling reactions and gel electrophoresis. The fusion protein was stable in vivo, as judged by the high yields of labeled material and the lack of detectable polypeptide products.

Our genetic selection for mutant fusion proteins deficient in biotinylation was based on the observation that basal expression of the ACP-BCCP hybrid gene of pTM4D increased the minimal concentration of biotin required for growth of strains blocked in biotin synthesis (bio strains). For growth in both liquid and solid minimal media (“Experimental Procedures”), bio strains harboring plasmid pTM4D required about 20 \(\text{nM}\) exogenous biotin, whereas strains carrying the vector plasmid (or no plasmid) grew readily on media containing about 1 \(\text{mM}\) biotin (data not shown).

**Isolation of Biotinylation-defective Mutations within the BCCP Biotin Domain**—Mutagenesis was localized to the BCCP-coding sequence by use of a mutagen specific to single-stranded DNA and gapped heteroduplexes in which only the BCCP DNA was single-stranded. Plasmid pTM4E DNA with 0.3-kb single-stranded loops containing the BCCP87 coding sequence (“Experimental Procedures”) was treated in vitro with methoxylamine, and the methylated DNA was rapidly purified and transformed directly into strain TM21 with selection for ampicillin resistance on RB plates (“Experimental Procedures”). This initial selection step was based on the increased biotin requirements of bio-lacZ hosts harboring plasmid pTM4D mentioned above. The biotin content of RB medium is only \(\sim 5 \text{ mM}\) (determined by bioassay on strain SA291 using the disc assay method of del Capillo-Campbell et al. (40)). Thus, strains carrying mutant plasmids that encoded fusion proteins that were poorly biotinylated would be expected to form colonies on RB medium since the defect in biotinylation would relieve the requirement for higher biotin concentrations (Fig. 2). In order to further sort these candidate colonies (and elim-
inate molecular siblings), each colony was then screened for its ability to repress the bio-lacZ reporter in a biotin-dependent manner using a radial streak assay (“Experimental Procedures”). This screen is based on the sophisticated regulatory system that controls biotin synthesis in *E. coli*. The BPL of *E. coli* functions not only as an enzyme but as the repressor regulating transcription of the biotin biosynthetic gene operon. *E. coli* BPL binds to the biotin operon operator only when complexed with biotinoyl-AMP, the product of the first half-reaction, whereas protein biotinylation consumes the biotinoyl-AMP and thus acts as an antagonist of DNA binding (4, 23). Therefore, overproduction of a biotin acceptor protein results in derepression of biotin operon transcription which in the present case is readily assayed by *β*-galactosidase production from the lacZ gene inserted into the biotin operon of the host strain chromosome (Fig. 2). (The bio mutation in these strains results from insertion of a promoter-less lacZ gene into the biotin operon such that the bioF gene is disrupted and the bio promoter drives the synthesis of β-galactosidase). As expected from prior studies using a different biotin acceptor protein (4, 23), induction of the parental fusion protein encoded by pTM4D/E resulted in an approximately 10-fold increase in bio-lacZ transcription at all biotin concentrations tested (data not shown) and was accompanied by greatly diminished biotinylation of endogenous BCCP (cf. Fig. 1B, lanes 1 and 2). Thus, overproduction of an exogenous biotin acceptor protein successfully competed with endogenous BCCP for the limited supply of biotin and also reduced occupancy of the bio operator by the BirA repressor.

This latter observation allowed development of the radial streak assay in which each mutant fusion candidate strain was placed on an X-gal indicator plate containing IPTG to induce expression of the fusion protein and then subjected to a gradient of biotin concentrations. At a given biotin concentration, strains that produced biotin domains deficient in biotin acceptance were expected to have lower levels of bio operon expression (hence shorter extents of blue cell growth due to X-gal cleavage by *β*-galactosidase) than the wild type strain carrying pTM4E, since the mutant proteins would compete poorly with the wild type (full-length) BCCP for the BPL-biotinoyl-AMP complex. Strains having decreased extents of bio operon induction (similar to that given by the host strain lacking pTM4E) were then further characterized by labeling with [3H]β-alanine and [3H]biotin.

Four independent mutagenesis experiments, in which heteroduplexes were exposed to methoxylamine for different periods, resulted in the identification of a total of 82 candidates that survived selection on low biotin and exhibited decreased repression of the bio-lacZ reporter. When examined by SDS-PAGE of [3H]β-alanine-labeled samples, only 42 of the 82 candidates expressed full-length fusion proteins. Fig. 3 shows the labeling observed in a representative sample of these isolates.
Characterization of mutated acetyl-CoA carboxylase-BCCP fusion constructs having defective biotinylation

The amounts of [3H]biotin and [3H]β-alanine incorporated into full-length fusion protein, in mutated acetyl-CoA carboxylase-BCCP iso-
lates which survived selection on low biotin and had reestablished bio-lacZ repression (Fig. 2), were assessed by SDS-PAGE of labeled cell extracts as described under "Experimental Procedures" (Fig. 3). ++ + + indicates wild type levels of incorporation, and − indicates extremely weak or undetectable incorporation. The mutations present in selected isolates were identified by sequence analysis. Only those mutations resulting in missense substitutions are shown. ts indicates the temperature-sensitive accB allele selected by Li and Cronan (3). NA, not applicable.

Expression and Purification of Biotin Domain Mutants—In order to evaluate the effect of individual amino acid substitutions on biotinylation, sequences encoding selected mutations were inserted individually into pTM53, a pET16 derivative used for production of wild type apoBCCP87 ("Experimental Procedures" and Ref. 32). The mutations were chosen on the basis of the frequency with which they had been selected following screening procedures (15). As expected, substitutions that resulted in apoproteins that showed evidence of proteolytic degradation during purification, as determined by SDS-PAGE confirmed both the expected amino acid substitutions and the biotinylation state of the purified protein samples (Table II).

Structural Stability of BCCP87 Mutants—Disulfide-linked dimers were detected using gels (Table II) and PAGE (data not shown) in the purified G133S, G133D, G143E, and V146I protein samples as seen previously in some preparations of wild type apoBCCP87 (15). This suggested that the availability of the single cysteine residue of the protein (Cys-116 in intact BCCP) could be used as a probe for structural alterations of the mutant domains. Since the recent NMR structures of apoBCCP87 show that Cys-116 is a buried residue that forms part of the hydrophobic core (14),2 the extent of disulfide dimer formation in the mutant apoproteins would reflect the extent to which the Cys-116 residues of two protein molecules were solvent-exposed and thus indicate a disruption of the native structure. Therefore, the tendency of the mutant apoproteins to form disulfide-linked dimers was investigated. Following reduction with DTT and gel filtration, samples were concentrated in the absence of reducing agent and analyzed for the presence of disulfide-linked dimers by non-denaturing PAGE (Fig. 5). Under these conditions, no dimer formation was observed for the wild type, E119K, or E147K mutant proteins. The V146I and G143E formed dimers to some extent, with 66 and 30%, respectively, of these proteins remaining monomeric. G133S was predominantly in the dimeric form, which persisted even when 10 mM DTT was present during concentration of the protein (data not shown). The rapid reformation of dimers of some mutant proteins precluded quantitative assessment of the accessibility of Cys-116 by reaction with sulphydryl reagents (15).

## Table I

| Allele | [3H] Biotin | [3H] β-alanine | Mutations |
|--------|-------------|----------------|-----------|
| Wild type | ++++ | ++++ | −         |
| 13 isolates | ++ | ++++ | E119K   |
| ts | − | NA | G133S   |
| 63 | ++ | + | G133D |
| 112 | − | + | G143R |
| 226 | ++ | + | S85F |
| 225 | − | ++++ | E119K, V146I |
| 279 | − | ++ | E119K, M123I |
| 46 | − | ++ | S85F, G133D |
| 62 | − | ++ | R84C, S85F, T94I |
| 55 | − | ++ | G89D, R93H, V105M |
| 58 | − | +++ | A76T, E119K, G143E, E147K |
| 121 | − | ++ | G89D, A120T, M124I, G143E |
| 79 | − | ++ | M87I, G89D, M121I, G143E |
| 69 | − | ++ | S79N, G89S, V118I, G143E, E147K |
| 100 | − | + | E71K, A76T, V88I, G89S, G143E |
| 12 | − | ++ | S85F, R93C, P102K, A129V |
|      |            |                | S132F, T134I, 145S |

![Fig. 3. [3H]Biotin and [3H]β-alanine labeling of the ACP-BCCP fusion protein in biotinylation-defective mutants.](image-url)
Previously, we used limited proteolysis to detect a subtle structural difference between apo- and holoBCCP87 (15). Together with the degradation observed during purification of several mutant proteins this suggested that proteolysis could be used as a sensitive indicator of possible structural perturbations caused by the amino acid substitutions. The apoBCCP87 mutant proteins were digested with trypsin under reducing conditions, to avoid the relatively rapid dimer formation characteristic of several of the mutant proteins. These digestions were carried out with 1 mM DTT (a 20-fold molar excess of reductant over protein) which was sufficient to maintain all of the mutant proteins in the monomeric form except apoG133S. The results shown in Fig. 6 indicate that the susceptibility of the apo form of the E119K and E147K proteins to trypsin digestion was equivalent to the wild type protein, whereas the V146I and G143E proteins showed increased sensitivity, and the G133S substitution resulted in extremely rapid degradation. Thus, the relative stabilities of the different mutant proteins were the same when evaluated using two different probes for structural alteration, i.e. disulfide dimerization and trypsin susceptibility.

Kinetics of Biotinylation—To evaluate the ability of the individual mutant proteins to act as substrates for E. coli BPL in vitro, it was first necessary to determine the optimal conditions for biotinylation of the wild type domain in a convenient assay system (“Experimental Procedures”). Assays of enzyme activity over the pH range 4.5–11.0 showed that maximal activity occurred in Tris-HCl, pH 8.0–8.5. Activity was higher in the Tris-HCl buffer than in MOPS or CAPS buffers, whereas sodium phosphate buffer was inhibitory. The enzyme was active over the range 5.5–10.0, with ~10% maximal activity at pH 5.5 and 50% at pH 10.0. As reported by other workers (41), K⁺ ions (50–100 mM) stimulated activity. The $K_m$ values for apoBCCP87, biotin, and ATP were 4.39 ± 0.37 μM, 0.49 ± 0.07 μM, and about 0.3 μM, respectively.

The BCCP87 mutant proteins were assayed at varying concentrations for the efficiency of biotinylation by BPL (Fig. 7). Kinetic constants derived from these data are given in Table III. The values for $k_{cat}/K_m$ for the different substrates show that the G143E protein had a similar affinity for the enzyme as wild type BCCP87, whereas the V146I protein was a slightly poorer substrate. The E147K substitution reduced the affinity about 3-fold. The E119K protein was an extremely poor biotinylation substrate. Biotinylation could only be detected when concentration of enzyme in the assay was increased 10-fold, and we were unable to obtain sufficiently high substrate concentrations to derive accurate kinetic constants for this protein. However, it was clear from assays carried out over the concentration range available (Fig. 7B) that the E119K mutation reduced the affinity of the biotin domain for the enzyme about 100-fold. The $K_m$ value determined for the G133S protein also could only be approximated and indicated that this protein was a poor substrate. The addition of 10 mM DTT to the assays to reduce the spontaneously formed G133S disulfide dimers increased the rate of biotinylation (Fig. 7A), whereas the presence of additional reductant had no effect on biotinylation of apo wild type BCCP87 (data not shown).

**DISCUSSION**

Our biological selection method allows facile isolation of mutant biotin accepting domains defective in interaction with BPL and is sufficiently robust that it can be applied to pools of randomly produced mutants. In addition, the ACP-BCCP fusion strategy enabled us to assess the expression, size, and stability of the fusions by an assay that did not depend on

![Fig. 4. Structure of the BCCP biotin-accepting domain showing isolated missense mutations.](Image)

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**Table II**

| Mutant  | Approprotein, mass calculated | Holoprotein, mass calculated |
|---------|------------------------------|----------------------------|
| E119K   | 9332.85                      | 9322.09 ± 0.91             |
| E147K   | 9332.85                      | 9322.81 ± 1.03             |
| G133S   | 9364.87                      | 18727.09 ± 3.06⁺           |
| G133D   | 9392.88                      | 18781.61 ± 3.35⁺           |
| G143E   | 9406.91                      | 9405.18 ± 0.40             |
| V146I   | 9348.87                      | 9346.49 ± 1.10             |

* Indicates a covalently linked dimer species.
biotinylation. Incorporation of both biotin and β-alanine readily eliminated chain termination mutants and allowed the extent of biotinylation relative to total fusion protein production to be easily determined. The mutated residues of these fusion proteins were decidedly non-random, and the most defective of the mutated proteins, E119K, was repeatedly isolated. It should be noted that, although the heteroduplex technique results in mutagenesis of both the coding and non-coding DNA strands, methoxylamine can mutagenize only those codons that contain cytosine or guanine bases. Moreover, mutations at the 3′ end of the genetic code. These parameters preclude mutagenesis of some positions of BCCP87 (such as the AAA codon that encodes the biotinylated lysine residue) and limit the possible amino acid substitutions at mutable positions. Therefore, some of the nonrandom nature of our mutant collection can be attributed to our mutagenesis protocol. Another bias lies in our construction of singly mutant domains from the isolates with multiple mutations. For example, we constructed the V146I domain since the E119K, V146I double mutant appeared more defective in biotinylation in vivo than the E119K mutant (Fig. 3), and the substitution seemed likely to alter the hydrophobic core of the protein. However, in vitro the V146I mutation had only a modest effect on biotinylation of the domain suggesting that the in vivo results were due to synergy between the two mutations rather than a simple additive effect. This is consistent with the location of the two mutated residues in the biotin domain (8, 14). The Co atoms of these two residues lie only 9.5 Å apart, and the structure is such that the altered packing required to accommodate isoleucine at position 146 could be

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**Fig. 5.** Disulfide-linked dimer formation. Samples of the BCCP87 mutant proteins that had been reduced with excess DTT, followed by removal of DTT by gel filtration chromatography (“Experimental Procedures”), were concentrated in the absence of reductant in a Centricon 10 device (Amicon) that had been pretreated with 1% bovine serum albumin according to the manufacturers’ instructions. The protein samples were run on non-denaturing PAGE in the presence of a Centricon 10 device (Amicon) that had been pretreated with 1% bovine serum albumin according to the manufacturers’ instructions. The wild type and mutant apoBCCP87 proteins were assayed over the indicated concentration range in the in vitro biotinylation reaction, and the data were analyzed as described under “Experimental Procedures.” The lines represent non-linear regression to the Michaelis-Menten equation using GraphPad Prism (“Experimental Procedures”). A, wild type ( ), E147K ( ■), G133S ( △), G133S + 10 mM DTT ( ▲), V146I ( ○), and G143E ( ●). B, the assays were carried out with a 10-fold higher enzyme concentration (125 nM) over 1 min with wild type BCCP87 ( ) and 15 min with E119K BCCP87 ( ●). Note the different axes for the two proteins.

**Table III**

Kinetic constants for the reaction of wild type and mutant BCCP87 proteins with E. coli BPL

| Protein substrate | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|------------------|-------|-----------|---------------|
|                  | µM   | s⁻¹       | µM⁻¹ s⁻¹      |
| Wild type        | 4.2 ± 1.6 | 0.16 ± 0.09 | 36.2 ± 7.1   |
| G133S            | 5.5 ± 1.8 | 0.15 ± 0.09 | 28.6 ± 18    |
| V146I            | 12.5 ± 0.6 | 0.23 ± 0.13 | 18.5 ± 10    |
| E147K            | 6.8 ± 1.8 | 0.084 ± 0.02 | 12.4 ± 0.06  |
| G133S            | 70    | ND        | ND            |
| E119K            | 400   | ND        | ND            |
propagated to the Glu-119 region. It should also be noted that all of the singly mutant proteins we examined retained some ability to accept biotin in vivo, and thus it seems difficult to completely block biotinylation of BCCP87 with only a single amino acid change. Indeed, the only mutant proteins we isolated that were completely defective in biotinylation in vivo each contained at least three amino acid substitutions (Table I).

The two mutant proteins with Glu to Lys substitutions (at residues 119 or 147) behave as authentic interaction mutants. The E119K protein is inactive as a substrate for BPL, whereas the E147K protein could be biotinylated, albeit poorly. Neither substitution seemed to affect the overall structure of the domain, as expected from the surface location of the parent lysine residues. Both proteins were indistinguishable from the wild type domain when assayed for disulfide dimer formation (Fig. 5) or trypsin resistance (Fig. 6). Therefore, we conclude that the E119K and E147K mutant domains are primarily defective in interaction with BPL. The surface of the biotin-binding pocket of the BirA protein (42) has several positively charged residues, and our data suggest that these residues may be involved in the correct positioning of the biotin acceptor protein at the catalytic site. Thus, alteration of the surface charge on the biotin domain surface that interacts with the ligase may affect the recognition between the two proteins. The notion of a matching of charged surfaces is consistent with mutational studies of the biotin domain of human propionyl-CoA carboxylase where changing the conserved PMP motif to PKP had a more pronounced effect on the efficiency of biotinylation than replacing all three residues with alanine (19). Several other observations suggest that charge maintenance may be particularly significant in the immediate vicinity of the biotinyl lysine, i.e. at Glu-119. A biotinylation consensus sequence selected from a largely randomized peptide library (43) has few residues that are strictly conserved with respect to the sequence around the biotinylation site in proteins. However, one of the derived constraints is for either Glu or Asp at the position equivalent to Glu-119 in BCCP (43), a finding consistent with the dramatically increased $K_m$ of the E119K mutant we observed. In addition, substituting Lys for either of Met residues that flank the biotinylated lysine greatly reduces biotinylation of the BCCP biotin domain in vivo (17). It is interesting that the functionally analogous interaction between lipoate ligase and the lipoil domains also appears to require charge conservation at the position equivalent to Glu-119. Substitution of lysine for the Glu found two residues upstream of the lipoilated Lys in many lipoil domains dramatically reduces lipoylation of both human glycine cleavage enzyme and E. coli pyruvate dehydrogenase (44, 45).

In contrast, our in vitro studies of the other purified proteins indicate that the defective biotinylation was the secondary consequence of defective domain folding. Substitution of other residues for the highly conserved glycine residues at positions 133 and 143 in BCCP destabilized the structure of the biotin domain. The G133S protein seems unable to fold stably (even under strongly reducing conditions), and thus the data we obtained most probably represent analysis of a mixture of the monomer and disulfide dimer forms of the protein. Gly-133 forms a turn between two $\beta$-strands on the face of the molecule opposite the biotinyl-Lys (8, 14).2 Since substitution of any amino acid side chain larger than Gly (or Ala) would produce a steric clash with the Ile-155 side chain and the Ser and Asp substitutions introduce a polar group into this hydrophobic region, we conclude that the increased $K_m$ reflects a defect in domain structure rather than an alteration directly affecting recognition by BPL. Indeed the G133S mutation is known to result in a temperature-sensitive accB phenotype in vivo (3).

The residual biotinylation we observed probably reflects interaction of BPL with a small and short-lived fraction of the protein that is properly folded. Expression of the G133S mutant protein in the presence of abnormally high levels of biotin ligase (from pCY216; Ref. 32) produced a biotinylated protein that largely behaved like the wild type domain during purification with no indication of proteolysis (data not shown). However, purified holoG133S BCCP87 did dimerize slowly during storage and was less stable to handling than the wild type holoprotein. Thus, it is apparent that, while apoG133S is highly unstable and subject to rapid dimerization and proteolysis, once biotinylated it becomes more stable and can function as an acetyl-CoA carboxylase subunit in vivo. Indeed, with the exception of the E119K protein, all of the mutant proteins could be produced in the biotinylated form in the presence of excess BPL and readily purified (Table II), consistent with the partial nature of the defects determined by the in vitro assays (Fig. 3 and Table III). The V146I protein also probably has a subtle structural defect. Although more stable than the proteins with substitutions of the glycine residues, the protein was less stable than the wild type protein, consistent with a role for Val-146 in structuring the hydrophobic core of the domain (8, 14).

The increased protease sensitivity of the structural mutants suggests that randomly produced BCCP biotin domain mutant proteins could be evaluated for structural alterations in crude cell lysates without protein purification. Furthermore, our in vitro analysis of the effect of the Val and Gly substitutions is consistent with straightforward predictions from the available structural information. Together, this suggests an approach that would allow efficient elimination of primarily structural defects to facilitate identification of additional interaction mutations.

Our kinetic analysis of the interaction of the wild type biotin domain with E. coli biotin ligase under steady state conditions gave kinetic constants that are not entirely consistent with those determined from initial rate measurements of the enzyme reaction (46). The values given here for the specificity constant $k_{cat}/K_m$ were in the same range as previously published data (46); however, the $K_m$ for apoBCCP87 was several orders of magnitude lower. It seems likely that this apparent discrepancy is due to the contribution of product dissociation, which is rate-limiting in the system of Nenortas and Beckett (46), to the $K_m$ determined here under steady state conditions. Indeed, more recent measurements of the initial rate in which the slower second phase of the reaction is included gives a $K_m$ for the reaction in the same range as the one determined in the present study.4 Similarly, the $\sim$10-fold higher $K_m$ for biotin is most probably due, in part, both to the different assay conditions and to the inclusion in the steady state measurements of additional rate constants following biotin binding.

It would be valuable to obtain values for the interactions of the wild type and mutant proteins by direct measurement of the BPL-domain interactions. However, these are very challenging experiments since the BPL species that binds the biotin domain is the BPL/biotinoyl-AMP complex rather than the uncomplexed protein (46). Hence, during the binding measurement the biotin domain will be rapidly converted from substrate to product, resulting in uninterpretable data. One approach to this problem would be to utilize a nonhydrolyzable analogue of biotinoyl-AMP, but no such analogue is known for this or any other acyl adenylate. A second approach would be to replace the substrate lysine residue with a residue unable to accept biotin. We have converted the substrate lysine residue to

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4 D. Beckett, personal communication.
a leucine residue, but we find that this protein is a poor inhibitor of biotinylation of the native domain and thus seems to be poorly recognized by the enzyme. Although we plan further attempts to find a suitable residue, it is possible that BPL recognition absolutely requires lysine at position 122.

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