Mutational Analysis of Protein Substrate Presentation in the Post-translational Attachment of Biotin to Biotin Domains*

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Biotinylation in vivo is an extremely selective post-translational event where the enzyme biotin protein ligase (BPL) catalyzes the covalent attachment of biotin to one specific and conserved lysine residue of biotin-dependent enzymes. The biotin-accepting lysine, present in a conserved Met-Lys-Met motif, resides in a structured domain that functions as the BPL substrate. We have employed phage display coupled with a genetic selection to identify determinants of the biotin domain (yPC-104) of yeast pyruvate carboxylase 1 (residues 1075–1178) required for interaction with BPL. Mutants isolated using this strategy were analyzed by in vivo biotinylation assays performed at both 30 °C and 37 °C. The temperature-sensitive substrates were reasononed to have structural mutations, leading to compromised conformations at the higher temperature. This interpretation was supplemented by molecular modeling of yPC-104, since these mutants mapped to residues involved in defining the structure of the biotin domain. In contrast, substitution of the Met residue N-terminal to the target lysine with either Val or Thr produced mutations that were temperature-insensitive in the in vivo assay. Furthermore, these two mutant proteins and wild-type yPC-104 showed identical susceptibility to trypsin, consistent with these substitutions having no structural effect. Kinetic analysis of enzymatic biotinylation using purified Met → Thr/Val mutant proteins with both yeast and Escherichia coli BPLs revealed that these substitutions had a strong effect upon $K_m$ values but not $k_{cat}$. The Met → Thr mutant was a poor substrate for both BPLs, whereas the Met → Val substitution was a poor substrate for bacterial BPL but had only a 2-fold lower affinity for yeast BPL than the wild-type peptide. Our data suggest that substitution of Thr or Val for the Met N-terminal of the biotinyl-Lys results in mutants specifically compromised in their interaction with BPL.

Biotin-dependent carboxylases are a ubiquitous family of enzymes that catalyze the transfer of carbon dioxide between metabolites using a biotin moiety as a carboxy carrier. These enzymes play key roles in essential metabolic processes (1). For example, pyruvate carboxylase catalyzes the generation of oxaloacetate, a precursor for the synthesis of glucose and fat as well as some amino acids and neurotransmitters (reviewed in Ref. 2). The biotin prosthetic group is covalently attached via the ε-amino group of a specific Lys residue within the protein (1), in a reaction catalyzed by biotin protein ligase (BPL; EC 6.3.4.15). The primary structure of the biotin domain shows a high degree of homology between a wide range of enzymes and species (3) and folds into an independent domain. Deletion studies on enzymes from both prokaryotes (4, 5) and eukaryotes (6, 7) have shown that biotin domains can be expressed as stable proteins of around 80 residues, which can be biotinylated both in vivo and in vitro (8). Furthermore BPLs from various sources have been found to be identified and biotinylate acceptor proteins from very different sources (1, 9), suggesting that all biotin domains fold into an essentially common tertiary structure and that the information required for association with BPL is present within this conserved structure.

The structures of two biotin domains have been determined: that of the Escherichia coli biotin carboxyl carrier protein (BCCP), a subunit of acetyl-CoA carboxylase (10, 11), and the 1.3 S subunit of the Propionibacterium shermanii transcarboxylase complex (TC) (12). These proteins are structurally homologous to the lipoyl domains of 2-oxo-acid dehydrogenase multienzyme complexes (13, 14), which undergo an analogous post-translational modification. They have a flattened β-barrel structure comprising two four-stranded β-sheets with the N- and C-terminal residues close together at one end of the structure. At the other end of the molecule, the biotinyl- or lipoyl-lysine resides on a highly exposed, tight hairpin loop between β-straonds four and five. The biotinyl-lysine is found in the motif Met-Lys-Met, which is highly conserved in all biotin domains (3). The precise positioning of the Lys appears to be important for recognition by BPL, as moving this residue by one position to either side of its normal position abolishes biotinylation (15). Additionally, the properties of the amino acid residues immediately surrounding the target Lys are important. Conservative substitutions of the flanking Met residues have revealed that these amino acids are not essential for interaction with BPL (6, 16), but do affect the carboxylation and carboxyl-transfer reac-

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The abbreviations used are: BPL, biotin protein ligase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PCR, polymerase chain reaction; PRS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; BCCP, biotin carboxyl carrier protein; SCR, structurally conserved region; TC, transcarboxylase complex.
tions of *P. shermanii* TC (17, 18). However replacement of the Met-Lys-Met motif of *E. coli* BCCP with the Asp-Lys-Ala motif characteristic of lipoyl domains, abolishes biotinylation by the bacterial BPL, BirA (19). Moreover, direct replacement of the lipoylation motif with the biotinylation motif in a lipoyl domain is not sufficient to specify biotinylation (20). It is evident that the specificity of biotinylation in *vivo* requires the interaction of a BPL with a structured protein substrate containing an exposed, precisely positioned Lys side chain.

Phage display is a powerful technique for investigating the interaction of proteins and peptides with other macromolecules. Polypeptides are expressed on the surface of filamentous bacteriophage as fusions to either the minor (gIIIp) or major (gVIIIp) coat proteins of the virion (21). The list of proteins that have been displayed on bacteriophage is ever increasing (see, for example, Refs. 22–24). Recently the biotin domain of *Klebsiella pneumoniae* oxaloacetate decarboxylase was displayed on the surface of a phage (25). Biotinylated phage were captured using avidin, indicating that the products of an enzymatic reaction can be specifically selected. Using phage display technology, proteins can be remodeled through mutagenesis and mutants with desirable phenotypes selected using an immobilized ligand. An understanding of the residues required for the function of the displayed protein can then be obtained by determining the primary structure of the selected mutants.

We have previously shown that the 104 C-terminal residues of pyruvate carboxylase 1 (YPc104) from *Saccharomyces cerevisiae* can be expressed as a stable biotin domain in *E. coli* (7). Furthermore, this peptide can be recognized and biotinylated in *vivo* by the *E. coli* biotin ligase, BirA. In the present study, we report the expression of biotinylated YPC104 on the surface of fd bacteriophage. Using phage display coupled with an in *vivo* selection in *E. coli*, we have investigated regions on the biotin domain molecule important for the interaction with BPL.

**EXPERIMENTAL PROCEDURES**

**Materials**

Oligonucleotides were purchased from Geneworks Ltd (Adelaide, Australia). The restriction sites in the oligonucleotides are underlined, and mutagenic changes are in bold. The sequences of the oligonucleotides are as follows: YPC104B, 5'-CATACCATGGCAATGAGAAGATTCTGTTGCTG-3'; YPC3'B, 5'-TAATCAAGCAGTATGCTTGCACAAAGGAAC-3'; YPC3'Leu, 5'-AAGATGGTGAATTCTTATCAGGCCATGGCCTATACGCTTA-3'; YPC104C, 5'-ACAGATTTCATGGCATTGGAAGAATTGGTTGCTG-3'; YPC104C, 5'-GAGATCCAGACCACTCGCAAGACTA-3'.

**DNA Manipulations and Sequencing**

Polymerase chain reactions, using 100 ng of oligonucleotides with 10 ng of template, were performed in 40 μl of 1× thermophilic buffer (Promega) containing 2.5–4 mM MgCl₂, 2 mM dNTPs, and 1 unit of Taq DNA polymerase with 30 cycles of denaturation at 95 °C for 1 min; annealing at 60–65 °C for 1.5 min, and synthesis at 72 °C for 1.5 min. Sequencing of all the constructs was performed using either the Sequenase Version II kit (Amersham Pharmacia Biotech) or ABI Prism Dye Terminator sequencing (PerkinElmer Life Sciences).

DNA encoding YPC104 was amplified using PCR with the oligonucleotides YPC104B and YPC3'C, with template pMW4A, which contains the cDNA for *S. cerevisiae* pyruvate carboxylase 1 (26). The primers introduced NcoI and PstI endonuclease restriction sites, respectively, facilitating the cloning of the 330-base pair product into the phagemid gPf4 (14, 27), a derivative of pHEN-1 (28). This generated the vector pFdBd-104. The biotinylated Yls residue at position 1135 in pyruvate carboxylase 1 was replaced with a Leu residue in the construct pFdBd-K1135L. The mutant was obtained by PCR using the oligonucleotides YPC3'Leu and YPC3'B and pFdBd-104 as the template. The mutated DNA was digested with endonuclease restriction endonucleases BclI and PstI and ligated into similarly treated pFdBd-104. Constructs pFdBd-104 and pFdBd-K1135L were transformed into *E. coli* XL1-Blue (Stratagene) for phage display.

DNA encoding YPC104 was cloned into the expression vector pKK223-3 (Amersham Pharmacia Biotech). Oligonucleotide YPC104C was employed, using PCR with YPC3'B and pFdBd-104, to add an EcoRI endonuclease restriction site onto the 5’ end of DNA encoding YPC104. The product was digested with EcoRI and PstI and cloned into similarly treated pKK223-3, generating the construct pC-104. This vector was subsequently digested with NcoI and PstI and ligated to the 330-base pair NcoI/PstI product liberated from pFdBd-K1135L, yielding the construct pC-K1135L. Proteins expressed from these vectors had an additional Met-Ala motif on the N terminus, as a result of cloning extra nucleotides at the 5’ region of the gene while introducing the NcoI restriction site.

**Expression of gIIIp Fusion Proteins**

XL1-Blue cells harboring either pFdBd-104 or pFdBd-K1135L were grown in 2YT supplemented with ampicillin (100 μg/ml), tetracycline (10 μg/ml), and 10 μM biotin. Overnight cultures were diluted 1:50 in fresh medium and grown to log phase at 37 °C, after which the expression of the fusion protein was induced with 0.02 mM IPTG for 16 h at 30 °C. Cell lysates were prepared as described by Chapman-Smith et al. (8) and analyzed by SDS-PAGE on 12% polyacrylamide gels (29). Biotinylated proteins were detected by Western blotting as described previously (8) and gIIIp fusion proteins detected using anti-gIIIp antibody (Mo Bi Tec, Göttingen, Germany).

**Biopanning**

Phagemids displaying yeast biotin domains were prepared essentially as described by Lucic et al. (27). Phagemids were panned using magnetic beads coated with Streptavidin (Dynal). For diagnostic panning, 30 μg of beads were incubated with 10⁹ phagemid in 1 ml of PBS, 0.1% BSA for 1 h at room temperature, captured using a magnet and washed 10 times in 1 ml of wash buffer (PBS, 0.1% BSA, 0.5% Tween 20). For panning of the library, 300 μg of beads, preblocked in PBS, 0.1% BSA, 0.025% Tween 20 for 1 h at room temperature, were mixed for 15 min at room temperature with 10⁸ phagemid particles equilibrated in 0.5 ml of wash buffer. The magnetic beads were captured and washed 5 times with 1 ml of wash buffer. After washing, beads were resuspended in PBS, 0.1% BSA and the bead suspension added to log phase XL1-Blue cells for 15 min at 37 °C. Bacteria infected with phagemids were detected by growth on LB media supplemented with 100 μg/ml ampicillin.

**Construction of Mutagenic Library**

DNA encoding YPC104 was amplified using error-prone PCR. Oligonucleotides YPC104B and YPC3'C and template pFdBd-104 were employed using the PCR conditions described above, except that dATP was 1 mM and 1 unit of Taq DNA polymerase from a batch known to be of low fidelity (Promega, batch no. 573 1805) were included in the reaction mix. PCR products were digested with NcoI and PstI, and 3.8 pmol of purified insert was incubated with 0.64 pmol of similarly treated pFdBd-K1135L and 2 units of T4 DNA Ligase (Roche Molecular Biochemicals). DNA was ethanol-precipitated in the presence of 0.13 M sodium chloride and 50 μg of carrier tRNA, and then dissolved in 20 μl of water. Electrocompetent XL1-Blue cells were prepared (30) and transformed with 1-μl aliquots of the DNA. The transformants were allowed to recover for 1 h in SOC medium (31) before an aliquot was withdrawn to estimate the size of the library. Phagemids from the library were prepared using the method of Gu et al. (32) with the following alterations: 2YT was supplemented with 100 μg/ml ampicillin and 10 μM biotin, and expression of the gIIIp fusion protein was induced with 0.02 mM IPTG. After 1 h of helper phage infection, 50 μg/ml kanamycin was added and the culture incubated at 30 °C overnight.

Phagemid particles were precipitated by incubation with polyethylene glycol (27) and stored at −80 °C in PBS.

**In Vitro Biotinylation of Mutant Biotin Domains on Phagemids**

Phagemids produced from the library were biotinylated with purified *E. coli* biotin ligase (a kind gift from D. Beckett, University of Maryland, College Park, MD) in buffer containing 40 mM Tris-HCl (pH 8.0), 3 mM ATP, 5.5 mM MgCl₂, 50 mM KCl, 5 μM biotin, 0.1 mM dithiothreitol, and 0.1% BSA. Phagemid particles (0.8–1.2 × 10⁷ colony-forming units) were reacted with 0.25 pmol enzyme at 37 °C for up to 24 h before panning. Phagemids were recovered from the reaction by precipitation with polyethylene glycol (27) and the pellet washed to remove free biotin before being resuspended in PBS for panning.
Mutational Analysis of a Yeast Biotin Domain

Compared in vivo biotinylation of the yPC-104 mutants was performed essentially as described previously by Val et al. (7). The mutants were expressed in E. coli TM21 cells by IPTG induction for 2 h from the pC-104-derived expression plasmids described above. Whole cell lysates were prepared using the method described by Chapman-Smith et al. (8). The expressed yPC-104 proteins was resolved from total cellular protein by fractionation on duplicate 15% (w/v) polyacrylamide gels under denaturing and reducing conditions. Protein was visualized by staining with Coomassie Brilliant Blue R250 and biotinylated protein detected by avidin alkaline phosphatase blot (8). The intensity of both the biotinylated band and the yPC-104 protein band were quantitated by laser densitometry and the extent of in vivo biotinylation, expressed as biotinylated protein divided by the total yPC-104 protein, in arbitrary units, for each peptide. This ensured that the quantitation of the extent of in vivo biotinylation was unaffected by any variation in expression levels or protein stability resulting from the introduced mutations.

Kinetic analysis of enzymatic biotinylation was performed using purified BPL and apo-yPC-104 peptides as described previously (9). $p$ values were calculated with two-tailed t tests using GraphPad Prism for Macintosh (GraphPad Software Inc, San Diego, CA). Peptides were purified from E. coli BL21(ADE3) cells harboring expression plasmids derived from pET16b (Novagen). These plasmids were constructed by digesting pET16b with NotI and PstI and introducing a similarly treated DNA fragment containing the mutant biotin domain coding regions, excised from the plasmids isolated from the in vivo selection.

Identification of Homologous Structures—Protein structures in the Protein Data Bank predicted to have homology to yPC-104 were identified by the threading method of Fischer and Eisenberg (34) as implemented on the UCLA structure-prediction server. The protein structures with compatibility (Z) scores higher than the confidence threshold of 5.0 $\geq 1.0$ were as follows: the lipoyl domain of pyruvate dehydrogenase complex from Saccharomyces cerevisiae (Ref. 35; IVW, Z $= 10.89$), the biotinyl domain of acetyl-CoA carboxylase from E. coli (Refs. 10 and 11; 1BDO, Z $= 9.65$), the lipoyl domain of A. vinelandii 2-oxoglutarate dehydrogenase (Ref. 36; GHJ, Z $= 8.25$) and the lipoyl domain of pyruvate dehydrogenase from Bacillus stearothermophilus (Ref. 14; LAB, Z $= 5.79$). From the individual sequence alignments, a consensus sequence alignment of yPC-104 to these proteins was generated (Fig. 5). Due to the lack of N-terminal regions homologous to that of yPC-104 (residues 1075–1098) in the identified structures, only residues 1099–1170 could be modeled. Secondary structure was predicted for residues 1075–1098 using the PHDsec program (37).

Homology Model Construction—Homology models of apo-yPC-104 and holo-yPC-104 residues 1099–1170 were constructed using the HOMOLOGY module of the INSIGHTII software (version 9.0; MSI, San Diego, CA). The structures listed above and the coordinates of the holo-BCCP domain (Refs. 10 and 11; 2BDO) and apo-BCCP domain (11, 38; 3BDO) were analyzed to identify the structurally conserved regions (SCRs) by iterative fitting of C$\alpha$ atom positions and analysis of hydrogen bonding patterns. For NMR ensembles a mean coordinate set, generated using MOLMOL (39) was used in the comparison process. The defined SCRs and loops are indicated in Fig. 5. For the models of holo- and apo-yPC-104, the SCRs and loop 1 coordinates were modeled on those of holo- and apo-BCCP, respectively. Coordinates for loop 2 in each model were based on loop 2 of the lipoyl domain of pyruvate dehydrogenase (Ref. 35; 1HYV).

Refrinement of Models—The two models were further refined by restrained simulated annealing using the program X-PLOR (40) employing the CHARMM force field (41). The restraints employed consisted of the backbone hydrogen bonds from the respective structure upon which the model was based (HN-O 1.7–2.0 Å and N-O 2.7–3.0 Å) and harmonic point restraints on the C$\alpha$ atoms tethering them to their positions in the initial models. This allowed the backbone to remain relatively fixed and the secondary structure to remain intact while side chain positions in the structure could be optimized to reduce steric clashes and promote intimate packing. Simulations were carried out in vacuo with the dielectric constant set to 10 to approximate solvent screening. Coordinate sets were cooled from 2000 to 100 K over 5 ps of dynamics, followed by 500 steps of conjugate gradient energy minimization.

RESULTS

Phage Display—The construct pFdBD-104 was generated, as described under “Experimental Procedures,” to express yPC-104 as a fusion to the gene III coat protein (gIIIp) of the filamentous bacteriophage fd in the E. coli supE host strain XL1-Blue (28). The two moieties were fused via a flexible glycine-rich linker containing an H64A subtilisin BPN’ protease sensitivity motif (33). Analysis of whole cell lysates by anti-gIIIp antibody and avidin blots showed that fusion protein expression was inducible with IPTG and the polypeptide was biotinylated in vivo by the bacterial BPL, BirA (data not shown). A second construct, pFdBD-K1135L, in which the codon for the biotinylated Lys was substituted with a codon for Leu, but otherwise identical to pFdBD-104, was produced to serve as a negative control in bioaffinity procedures. Expression of this gIIIp fusion protein was detected in whole cell lysates, as described under “Experimental Procedures.” However, as expected, avidin blot analysis showed that this mutant polypeptide was not a substrate for the in vivo biotinylation reaction (data not shown).

Phagemids displaying the fusion proteins were prepared under conditions known to allow monovalent display (28), captured using magnetic beads coated with streptavidin, and used to infect male E. coli cells as described under “Experimental Procedures.” Those phagemids bound to the beads were mixed directly with E. coli cells and transduction assayed by growth in the presence of ampicillin. The efficiency of panning was such that a single panning step produced a 5,000-fold enrichment of the biotinylated yPC-104 phagemids over the null phagemid, Bluescript. Similarly low recoveries of phagemids displaying the nonbiotinylated mutant expressed from lys 1135→Leu phagemids and the null control were observed. Thus, the interaction of the phagemids with the streptavidin-coated beads, and their subsequent capture, was specific for the presence of a biotin group covalently attached to the phagemids via lys 1135.

Construction of yPC-104 Mutant Library—Using phage display and the in vivo selection described under “Experimental Procedures,” we devised a novel method for investigating the protein; protein interaction between a biotin domain and a BPL. First, a library of mutant biotin domains were displayed on the surface of bacteriophage, and those members that were products of enzymatic biotinylation were specifically captured with streptavidin. This step enabled the removal of mutations inducing gross structural changes that abolished biotinylation. Second, an in vivo genetic selection was employed to segregate the remaining library members specifically into a pool of mutants having decreased affinity for BirA. Briefly, this selection relies on competition by an efficiently biotinylated overexpressed protein with endogenous BCCP for a limited pool of free biotin when expressed in a biotin auxotroph. Expression of mutants that are poor substrates for BirA results in biotinylations of BCCP, producing functional acetyl-CoA carboxylase and allowing cell growth. We have previously used this selection to isolate mutations in the biotin domain of E. coli BCCP having decreased affinity for BirA (42).

A phage display library encoding mutated yPC-104 was constructed by error-prone PCR, using Taq DNA polymerase and limiting the availability of dATP in the reaction, as described under “Experimental Procedures.” After infecting the transformed bacteria with helper phage, a phagemid library of the INSIGHTII software (version 9.0; MSI, San Diego, CA). The two moieties were fused via a flexible glycine-rich linker containing an H64A subtilisin BPN’ protease sensitivity motif (33). Analysis of whole cell lysates by anti-gIIIp antibody and avidin blots showed that fusion protein expression was inducible with IPTG and the polypeptide was biotinylated in vivo by the bacterial BPL, BirA (data not shown). A second construct, pFdBD-K1135L, in which the codon for the biotinylated Lys was substituted with a codon for Leu, but otherwise identical to pFdBD-104, was produced to serve as a negative control in bioaffinity procedures. Expression of this gIIIp fusion protein was detected in whole cell lysates, as described under “Experimental Procedures.” However, as expected, avidin blot analysis showed that this mutant polypeptide was not a substrate for the in vivo biotinylation reaction (data not shown).

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restricted to two rounds to minimize the possibility of losing low affinity mutants over multiple rounds. After two rounds of panning, the phagemids were used to infect XL1-Blue cells and plasmid DNA was extracted.

Selection of Biotinylation Defective Mutants in Vivo—DNA encoding the mutant biotin domains was excised from the phage display vector and cloned into the expression vector pC-104 for the genetic selection. The ligation products were introduced into the biotin auxotroph E. coli strain TM21 (42) and grown on media containing IPTG, ampicillin and limited biotin for selection of biotin domain mutants in vivo. Transformants were replica-plated onto both selective and nonselective media for growth phenotype analysis. Approximately 80,000 colonies were screened in this manner, and a wide range of colony sizes was observed on the selective media. Since growth under these conditions results from expression of a poorly biotinylated protein (42), plasmid DNA was isolated from colonies showing the best growth. Eighteen isolates giving the expected restriction pattern were further analyzed by DNA sequencing, and point mutations were detected in all clones. These data (Fig. 1) documented 12 unique single missense mutations. In addition, three silent mutations and two double missense mutations were isolated.

Analysis of Biotinylation in Vivo by BirA—The mutants isolated using phage display and the in vivo selection were analyzed to determine the ability of the peptides to function as substrates for BirA in vivo at 37 °C, as described under “Experimental Procedures.” Since the extent of biotinylation was determined as a function of the amount of biotinylated protein relative to total yPC-104 protein for each mutant protein analyzed, our quantitation of biotinylation was unaffected by any variation in expression levels or protein stability resulting from the introduced mutations. The extent of biotinylation for each of the mutants was expressed relative to wild-type yPC-104, quantitated in the same manner (Fig. 1). With the exception of the His1102 → Arg and His1117 → Arg substitutions, mutations in the region of the molecule proposed to form the structured biotin domain caused a decrease in biotinylation. As expected, the Lys1135 → Leu substitution abolished biotinylation and was included in the assays as a negative control. Two mutations were found at the highly conserved Met1134 residue adjacent to the biotinyl-Lys. Substitution of this residue with Val or Thr caused similar decreases in biotinylation to 69% and 74%, respectively, of wild-type levels. Substitution of Pro at Ser1141 or Ser1162 and the Ile substitution at Phe1152 gave decreases in biotinylation from 40% to 65% of wild-type levels. Two bands were observed on the avidin blot for the latter mutant (data not shown), suggesting the protein was susceptible to proteolysis by E. coli enzymes in vivo. Substitution of Val residues with Ala at positions 1148 and 1166 decreased biotinylation to around 50%, whereas this substitution at position 1116 did not affect biotinylation in this assay.

Several mutations were detected in the amino-terminal region of yPC-104 lying outside the structurally conserved biotin domain. Substitution of Ala1081 with Pro reduced biotinylation to 52% and the double mutant Arg1083 → Gly/Lys1086 → Ile to 82%, whereas Arg1083 → Gly alone had little effect. Individual mutations from the His1102 → Arg/Met1107 → Thr double mutant, which showed 46% of wild-type biotinylation, were cloned into separate vectors and analyzed but neither single mutation had a large effect on biotinylation (Fig. 1) suggesting that the large decrease observed for the double mutant was the result of synergistic effects.

Temperature Sensitivity of yPC-104 Mutants—Temperature-sensitive mutations of BCCP have been shown to affect the conformation of the biotin domain and, as a result, substrate recognition (5, 42). To investigate the possibility that the amino acid substitutions in yPC-104 induced structural alterations, the in vivo biotinylation assay was carried out at a lower temperature for selected mutants (Fig. 2). Substitutions at all positions investigated, with the exception of Met1134, showed an increase in the extent of biotinylation at 30 °C. Substrates Phe1152 → Ile and Val1166 → Ala were comparable to the wild-type peptide at the lower temperature. Furthermore, the proteolytic product of Phe1152 → Ile observed at 37 °C was not evident at 30 °C. The efficiency of biotinylation increased by 31% for Val1166 → Ala, 40% for Ser1141 → Pro, and 67% for Ser1162 → Pro. We conclude that point mutations of yPC-104 that were temperature-sensitive most likely altered the conformational stability of the biotin domain, rendering it a less favorable substrate for BirA at the higher temperature. In contrast, substitution of Met1134 with either Val or Thr were not temperature-sensitive mutations. The extent of biotinylation of Met1134 → Thr and Met1134 → Val was essentially the same at both 30 °C and 37 °C, suggesting that these substitutions did not result in structural alteration.

Predicted Structure of yPC-104—To assist in interpreting the results obtained from in vivo analysis of apo-yPC-104 mu-
tants, we constructed a molecular model of the yeast biotin domain (Fig. 3). This model was based on the solution structures of apo-BCCP-87 and several known structurally analogous lipoyl domains (see “Experimental Procedures”). We also predicted the structure of holo-yPC-104 modeled upon holo-BCCP, with the apo- and holo-yPC-104 structures being essentially identical. For comparison purposes, the first member of the apo-BCCP (3BDO) and holo-BCCP (2BDO) NMR ensembles were subjected to identical “refinement” protocols. For both yPC-104 and BCCP models, 10 simulations were carried out and the five final models with the lowest overall energy were retained for analysis (Table I). All the model ensembles are tight, in terms of atomic root mean square deviation values and energetics, and one model is as good as any other. The deviation of model BCCP coordinates from the starting experimental coordinates is similar to the root mean square deviation values of model BCCP coordinates from the starting experimental basis. In addition, yPC-104 has a much higher charge density on its surface and thus the potential for many more unfavorable interactions.

From sequence alignments, shown in Fig. 4, the eight β-strands of apo-yPC-104 were defined as structurally conserved regions (β1 Leu1101–Ala1105, β2 Val1110–Ile1111, β3 Leu1121–Ile1122, β4 Pro1127–Ser1132, β5 Glu1137–Ser1141, β6 Gly1146–Val1151, β7 Glu1157–Val1159, β8 Asp1163–Leu1168). Like other biotin and lipoyl domains, the molecule is stabilized by a central hydrophobic core. In addition, a hydrophobic patch exists on the surface of the molecule formed by the side chains of their BCCP-based starting coordinates (apo-yPC-104, 1.26 ± 0.04 Å; holo-yPC-104, 1.17 ± 0.04 Å) and have energy values comparable to those of the BCCP models for all terms except the harmonic term and the electrostatic term. The difference in the magnitudes of harmonic terms between the yPC-104 and BCCP models is interesting and suggests that the BCCP backbone atom positions suit the yPC-104 models very well. The electrostatics of the BCCP models are better than that of the yPC-104 models, as expected since the charged side chain positions in the BCCP models have some experimental basis. In addition, yPC-104 has a much higher charge density on its surface and thus the potential for many more unfavorable interactions.

![FIG. 2. Temperature sensitivity of yPC-104 mutants.](image)

**TABLE I**

| Measures of coordinate deviations, energies, and stereochemical properties of the models and the NMR ensembles |
|---------------------------------------------------------------|
| **Apo-yPC** | **Holo-yPC** | **Apo-BCCP** | **Holo-BCCP** |
| --- | --- | --- | --- |
| **Energiesa** | | | |
| Overall | 53 ± 14 | 80 ± 9 | 170 ± 6 | 128 ± 7 |
| Bonds | 22.4 ± 0.3 | 22.6 ± 0.7 | 30.2 ± 0.3 | 30.7 ± 1.1 |
| Angles | 122 ± 3 | 125 ± 1 | 147 ± 5 | 141 ± 4 |
| Dihedrals | 258 ± 3 | 264 ± 4 | 319 ± 8 | 311 ± 6 |
| Improper | 3.7 ± 0.1 | 3.4 ± 0.1 | 3.7 ± 0.3 | 3.2 ± 0.2 |
| VDW | −349 ± 7 | −344 ± 4 | −348 ± 4 | −351 ± 4 |
| Distance | 0.23 ± 0.02 | 0.30 ± 0.07 | 0.09 ± 0.02 | 0.17 ± 0.08 |
| Harmonic | 4.4 ± 1.1 | 3.8 ± 0.9 | 44.9 ± 2.6 | 33.5 ± 3.0 |
| B.B. root mean square deviationb | 0.27 ± 0.10 | 0.27 ± 0.11 | 0.30 ± 0.07 | 0.34 ± 0.07 |
| H.A. root mean square deviationc | 0.63 ± 0.17 | 0.66 ± 0.20 | 0.75 ± 0.17 | 0.86 ± 0.20 |
| **Ramachandran propertiesd** | | | |
| Most favored | 67.7 | 72.0 | 69.6 | 74.9 |
| Allowed | 26.0 | 23.7 | 23.8 | 19.1 |
| Generous | 3.7 | 3.7 | 4.3 | 3.6 |
| Disallowed | 2.6 | 0.6 | 3.2 | 2.4 |

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*a Energy terms in kcal mol⁻¹ calculated in X-PLOR using default CHARMM22 parameters and with a dielectric constant of 10. The Distance term refers to the violation of hydrogen bond restraints (treated as nuclear Overhauser effect restraints) not hydrogen bond energies which are implicit in the VDW and Elec terms.

*b Backbone (N,Cα,C′) pairwise root mean square deviation of the five refined models.

*c Heavy (non-hydrogen) atom pairwise root mean square deviation of the five refined models.

d Calculated for each residue in each conformer (not angular averages) using the in-house program ANGORDER. ANGORDER uses the Ramachandran region defined by Morris et al. (47), which is the same as employed by the PROCHECK suite of programs (48). Values are percentages of non-Gly and non-Pro residues and exclude the N- and C-terminal residues.
of Val1151, Phe1152, Val1153, and Leu1164.

Interpretation of Mutational Results—We used temperature sensitivity to assess the structural stability of yPC-104 mutants. The three residues where Val → Ala mutations were isolated, Val1116, Val1148, and Val1166, contribute to the hydrophobic core of the predicted structure and form contacts with each other (Fig. 5). It is reasonable to conclude that replacement of the larger Val side chain with Ala distorts the packing arrangement of the hydrophobic core, decreasing protein stability at the higher temperature. Our modeling suggests that the two temperature-insensitive mutants, Met1134 and the adjacent Met1134, are shown. The figure was generated using Molspec, version 2.1.1 (46).

Loss of favorable interactions between them, is likely to alter the way the loop is presented to the enzyme. Met1134 resides on this loop adjacent to the critical lysine (Fig. 5); however, it does not contribute to the hydrogen bonding in the hairpin, and so its substitution is unlikely to destabilize this turn.

The secondary structure of the 23 N-terminal amino acids of yPC-104, lying outside the structured biotin domain, was predicted using the PHDsec program (37). Residues Lys1135, Val1151, Phe1152, Val1153, and Leu1164 were predicted to form an α-helix (Fig. 4). The point mutations Ala1081 → Pro and Arg1083 → Gly mapped to residues forming this α-helix. Both substitutions introduced residues known to disrupt α-helices, consistent with the notion that this region of the molecule may indeed be structured.

The mutant Phe1152 → Ile showed temperature sensitivity in the in vivo biotinylation assays. This suggests that the hydrophobic patch on the surface of yPC-104, containing Phe1152, plays a role in the stability of the domain. Furthermore, this mutant has a higher susceptibility to proteolysis, supporting the involvement of Phe1152 in an interaction stabilizing the domain. In the crystal structure of BCCP, the corresponding residue (Leu1152) interacts with another hydrophobic residue (Ile128) found in the short N-terminal tail outside of the structured domain (10). We propose that the hydrophobic patch on the surface of yPC-104 may interact with the N-terminal region of yPC-104, possibly through a hydrophobic region on the predicted α-helix, and that the change to Ile disrupts this interaction. We conclude that the observed temperature sensitivity of the mutants we have isolated is consistent with their proposed contribution within the structured biotinyl domain of yPC-104.

Kinetic Analysis of Biotinylation in Vitro—Wild-type yPC-104 and the two temperature-insensitive mutants, Met1134 → Val and Met1134 → Thr, were purified as unbiotinylated domains (9) for further investigation. Molecular mass determination by electrospray mass spectrometry confirmed the biotinylation state and expected amino acid substitution of the purified proteins. The peptides were 131 mass units less than the expected molecular mass (yPC-104: mass calculated 11,422.3, mass determined 11,292), suggesting the N-terminal Met (131.2) was excised in vivo. The ability of these biotin domains to function as substrates for BirA was assessed.
**FIG. 6. In vitro biotinylation assays.** Purified apo-biotin domains were assayed with BirA (A and B) or yBPL (C and D) using the conditions described by Chapman-Smith et al., (42) and Polyak et al. (9), respectively. The activity of the enzymes was determined over the concentration range of apo-biotin domains indicated on the graphs. The substrates analyzed were yPC-104 (■), Met\(^{1134} \rightarrow \) Val (▲), Met\(^{1134} \rightarrow \) Thr (●), and BCCP-87 (○). In the graph in A, 10-fold more BirA was included in the reactions for the two yPC mutants. The lines represent the nonlinear regression to the Michaelis-Menten equation using GraphPad Prism for Macintosh (GraphPad Software Inc., San Diego, CA).

| Table II | Kinetic constants for the reaction of biotin domains with E. coli BirA and yeast BPL |
| --- | --- |
| Enzyme | Substrate | \( K_m \) (\( \mu M \)) | \( k_{cat} \) (\( s^{-1} \)) | \( k_{cat}/K_m \) (\( \times 10^{-3} \) M\(^{-1}\) s\(^{-1} \)) |
| BirA | BCCP-87 | 3.2 ± 0.5 | 0.09 ± 0.02 | 28.5 ± 0.01 |
| yPC-104 | Met\(^{1134} \rightarrow \) Val | >200 | ND | ND |
| yPC-104 | Met\(^{1134} \rightarrow \) Thr | >200 | ND | ND |
| Yeast BPL | yPC-104 | 1.0 ± 0.2 | 0.6 ± 0.1 | 6.6 ± 0.4 |
| Met\(^{1134} \rightarrow \) Val | 2.0 ± 0.2 | 0.6 ± 0.2 | 2.9 ± 0.6 |
| Met\(^{1134} \rightarrow \) Thr | >200 | ND | ND |
| BCCP-87 | 11 ± 1 | 0.7 ± 0.1 | 0.7 ± 0.01 |

using steady state kinetics under conditions that were optimal for biotinylation of BCCP-87 (Ref. 42; Fig. 6, A and B). BCCP-87 was the best substrate for the bacterial enzyme, whereas the affinity of the wild-type yeast biotin domain for BirA was ~10-fold lower (Table II). Similar \( k_{cat} \) values were observed for these two substrates, suggesting that the deficiency in biotinylation was not due to changes in the rate of catalysis. Biotinylation of the two yeast domain mutants could only be detected upon increasing the ligase concentration in the assay by 10-fold. Since we were unable to obtain sufficiently high concentrations to obtain accurate kinetic constants for the two mutant proteins, \( K_m \) was estimated from a double-reciprocal plot at low substrate concentration. This indicated that both mutants had a \( K_m \) for BirA at least 10-fold higher than wild-type yPC-104 (Table II).

When the proteins were assayed with yeast biotin ligase under conditions that were optimal for biotinylation of yPC-104 (Fig. 6, C and D; Table II), the wild-type yeast biotin domain was the preferred substrate, with the affinity for BCCP-87 being 10-fold lower, as described previously (9). The Met\(^{1134} \rightarrow \) Val mutant, which was a poor substrate for BirA, displayed only a slightly lower affinity than wild-type yPC-104 (2-fold, \( p = 0.0002 \)). As was observed with BirA, Met\(^{1134} \rightarrow \) Thr was also a poor substrate for the yeast enzyme displaying ~20-fold higher \( K_m \) than wild-type, estimated from a double reciprocal plot at low substrate concentration. With yBPL as with BirA, similar rates of biotin transfer (i.e. \( k_{cat} \)) were observed for the yeast and bacterial substrate proteins. Thus, the differences in affinity (i.e. \( k_{cat}/K_m \)) arise primarily from differences in \( K_m \) values.

**TrpY Susceptibility of Met\(^{1134} \rightarrow \) Thr and Met\(^{1134} \rightarrow \) Val**—As a second probe for the structural integrity of the Met\(^{1134} \) mutants, the purified apo-proteins were subjected to limited trypsin digestion. We have previously used susceptibility to trypsin digestion to investigate the effect of point mutations on the structure of a biotin domain (42). Digestion of wild-type yPC-104 analyzed by SDS-PAGE showed an initial rapid cleavage yielding a product with an apparent molecular mass of 11 kDa (Fig. 7). A second, slower cleavage generated a stable product, which migrated at around 10 kDa. N-terminal sequencing of this cleavage product revealed two scissile bonds between Arg\(^{1083} \)–Ser\(^{1084} \) and Lys\(^{1086} \)–Val\(^{1087} \) at or near the C-terminal end of the proposed o-helix in the N-terminal extension. This product containing the C-terminal structured biotin domain was relatively resistant to further proteolysis, as it was the major species even after 5 h of treatment (data not shown). The two purified mutant apo-biotin domains were cleaved by trypsin at the same rate as the wild-type protein and generated the same products (Fig. 7). These results indicate that substitution of Met\(^{1134} \) with either Thr or Val did not induce significant conformational changes to the structured domain.

**DISCUSSION**

In all biotin-dependent enzymes, the residues immediately flanking the biotin-accepting Lys are invariably Met. Mutation of these residues in both the biotin-accepting subunit of P. shermanii transcarboxylase (17) and in synthetic peptides (18) has revealed that these Met residues are required for the carboxylation of biotin, the first stable intermediate of the biotin carboxylase reaction. It has been demonstrated that the flanking Met residues are not essential for biotinylation by BPLs since proteins having conservative substitutions are still recognized as substrates (6, 16). In the present report, we isolated two classes of mutations to the biotin domain of pyruvate carboxylase from Saccharomyces cerevisiae by screening a library of randomly mutated polypeptides using phage display...
and a genetic selection in *E. coli* that permits the isolation of peptides with a lowered affinity for bacterial BPL. The first class of mutations isolated contained two distinct, conservative substitutions of the Met residue immediately N-terminal of the target Lys. One mutant, Met<sub>1134</sub> → Val, was a poor substrate for both the bacterial and yeast BPLs. In contrast, although the Met<sub>1134</sub> → Thr substitution was also a poor substrate for the bacterial BPL, it was only a 2-fold poorer substrate for the yeast BPL than the wild-type protein (Fig. 6, Table II). Our data show that there are subtle differences in the active sites of BPLs from various species which tolerate mutations to the flanking Met to varying degrees. Furthermore, it has been known for some time that BPLs display cross species reactivity (1, 3). Here we provide the first kinetic evidence that BPLs biotinylate heterologous biotin domains with a lower efficiency than they do their native substrates. Both the yeast and bacterial BPLs displayed at least a 10-fold greater affinity for their homologous substrates over heterologous biotin domains (Fig. 6, Table II). Together these data indicate that, although BPLs catalyze the same reaction, there are distinct differences between the two enzymes for optimal substrate recognition.

It is apparent that BPLs recognize and interact with a structured biotin domain (13, 42). The second class of mutants isolated using our selection technique mapped to residues some distance from the target lysine. All mutants analyzed belonging to this class were temperature-sensitive, suggesting that these amino acid substitutions induced conformational changes to the folded domain, making it a compromised substrate at 37 °C. This finding is consistent with our previous work, which has demonstrated that several mutations located at a distance from the target lysine of the *E. coli* BCCP biotin domain reduce biotinylation as a consequence of structural alterations (42). To facilitate interpretation of our mutant data, we have produced a molecular model of yPC-104 based upon known biotin domain structures (Fig. 5). Our models of yPC-104 differ from that described by Brocklehurst and Perham (13) primarily due to the availability of additional experimentally determined structures from which to construct a more rigorous model of the protein. Using the BCCP coordinates as a guide allowed us to better model the loop between β-strands 1 and 2, and thus our positioning of β-strand 1 differs in register by two residues. We also found no requirement for lengthening the loop between β-strands 7 and 8, and thus our alignment of β-strand 8 differs in register by one residue position.

The mutations obtained in the present study were mapped onto our model (Fig. 5). All the temperature-sensitive mutations, predicted to induce conformational changes to the molecule, were at residues implied to be necessary for defining protein structure. In addition, the molecular modeling of the yeast biotin domain highlighted some novel properties, especially in the N-terminal region outside of the structured domain. This region contains a predicted α-helix with three hydrophobic residues (Ile<sup>1078</sup>, Val<sup>1080</sup>, and Ala<sup>1081</sup>), which may function as a cap, packing against the surface of the biotin domain via the hydrophobic surface involving residues Val<sup>1151</sup>, Phe<sup>1152</sup>, Val<sup>1153</sup>, and Leu<sup>1164</sup>. We have previously shown that expression of a peptide lacking the 23 N-terminal amino acid residues is a 6-fold less favorable substrate in *in vivo* biotinylation assays than the 104 residue peptide (7). Without the N-terminal extension, the domain was unstable during purification and displayed a marked tendency to form high molecular weight aggregates on gel filtration chromatography. These observations are consistent with our structural predictions and mutant data, which suggest that the N-terminal extension plays a role in stabilizing the overall domain structure, rendering the molecule a better substrate for BirA.

The BCCP domain contains a protruding “thumb” between strands β2 and β3 (10, 11) that is not present in the structurally heterologous lipoyl domains (Fig. 3 & 4). Interestingly, this thumb contacts the biotin moiety in both the crystal (10) and solution (11) structures of BCCP. The sequence of amino acids which form this thumb is absent in most other biotin domains including *P. shermanii* TC (1, 3). Furthermore, determination of the structure of the 1.3 S subunit of *P. shermanii* TC has revealed that no thumb structure is present in the domain (12) and the biotin moiety does not contact the protein (43). In this regard, our predicted fold of yPC-104 more closely resembles the structure of the 1.3 S subunit of TC. It is possible that the absence or presence of the thumb on the biotin domain contributes to the 10-fold reduction in affinity shown by the bacterial and yeast BPL with a heterologous substrate.

Biotinylation and lipoylation *in vivo* by BirA and the two lipoyl attachment enzymes, LpLA and LpLB, respectively, occur in a highly specific manner despite the striking structural similarity of the accepting domains (19, 20). It has been demonstrated that the thumb structure of BCCP functions to inhibit lipoylation, as a thumb deletion mutant of BCCP-87, which still functioned as a substrate for BirA, was lipoylated *in vivo* and *in vitro* by LpLA (19). The lipoyl domains lack the thumb, having three residues which span the region corresponding to the base of the thumb, but contain an extended surface loop between β-strands 1 and 2, which is close in space to the lipoyl-lysine (Fig. 3 and 4; Refs. 14, 35, and 36). The residues forming the surface loop are not required for *in vivo* lipoylation, but instead are necessary for the reductive acetylation of the prosthetic group (44). However, as biotinylation of the lipoyl domain does not occur in *E. coli in vivo* (20), this surface loop may also function to inhibit recognition by bacterial BPL. In the BCCP domains, this middle finger loop is replaced by two residues spanning the region (Fig. 4). The yPC-104 domain lacks both the thumb (loop 2, Figs. 3 and 4) and the extended middle finger loop (loop 1, Figs. 3 and 4), further suggesting that structural differences govern substrate recognition by yeast and bacterial BPLs.

The selection procedure we used was established using *E. coli* BCCP-87 (42). We showed that, despite BirA having a 10-fold higher affinity for its native substrate over yPC-104 *in vitro*, high level expression allowed the *in vivo* selection to be adapted for mutational analysis of the yeast protein. Although no wild-type DNA molecules were isolated after selection, several silent mutations were obtained. Mutations that destabilize mRNA, reduce translation efficiency, or interfere with protein stability would all decrease the selection pressure on the host.

Fig. 7. Tryptic sensitivity of apo-yPC-104 mutants. Proteins were treated with trypsin using the conditions described by Chapman-Smith et al. (42). Samples were taken at various time intervals indicated above and analyzed by SDS-PAGE. The panels show the digestion products of apo-yPC-104 (A), apo-Met<sup>1134</sup> → Thr (B), and apo-Met<sup>1134</sup> → Val (C).
These aspects of our selection procedure account for those mutants obtained in the selection that functioned as wild-type-like substrates in the in vivo biotinylation assay, since this assay corrects for variations in expression levels. It should be noted that the relative affinities of the mutant peptides obtained from the in vivo and in vitro biotinylation assays are a reflection of the different assay conditions. In the in vivo assay that measures accumulated product, factors such as the concentration of enzyme, ATP, and biotin cannot be controlled. On the other hand, the in vitro biotinylation assay measures initial velocity under steady-state conditions using purified components with optimal, saturating concentrations of ATP and biotin.

The enzymatic reaction catalyzed by BPLs proceeds through two partial reactions. In the first partial reaction, ATP and biotin bind BPLs in an ordered manner (9, 45) and the carboxyl group of inert biotin is activated by the addition of an adenylate group. In the second partial reaction, nucleophilic attack upon activated biotin by the amine group of the Lys in the protein substrate results in the transfer of biotin from the adenylate onto the apo-biotin domain, with AMP behaving as the leaving group (45). The Lys side chain must be precisely positioned into the active site of BPL to bring the electron donating nitrogen close enough to the reactive biotinyl-5'-AMP moiety to permit the chemical reaction. Thus, the biotin domain functions as a protein scaffold that presents the biotin-accepting Lys to the active site of BPL to bring the electron donating nitrogen close enough to the reactive biotinyl-5'-AMP moiety to permit the chemical reaction. This, the biotin domain functions as a protein scaffold that presents the biotin-accepting Lys to the active site of BPL to bring the electron donating nitrogen close enough to the reactive biotinyl-5'-AMP moiety to permit the chemical reaction.

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