Stabilization of the Biotinoyl Domain of *Escherichia coli* Acetyl-CoA Carboxylase by Interactions between the Attached Biotin and the Protruding “Thumb” Structure*

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We previously reported (Chapman-Smith, A., Forbes, B. E., Wallace, J. C., and Cronan, J. E., Jr. (1997) *J. Biol. Chem. 272*, 26017–26022) that the biotinylated (holo) species of the biotin carboxyl carrier protein (BCCP) biotinoyl domain is much more resistant to chemical modification and proteolysis than the unbiotinylated (apo) form. We hypothesized that the increased stability was due to a conformational change engendered by interaction of the domain with biotin protein ligase, the enzyme that attaches the biotin moiety. We now report that a BCCP-87 species to which the biotin moiety was attached by chemical acylation rather than by biotin protein ligase showed the characteristically greater stability of the holo biotinoyl domain. This result demonstrates that our hypothesis was incorrect; the attached biotin is solely responsible for the increased stability. The bacterial and chloroplast multisubunit acetyl-CoA carboxylases are unusual in that the highly symmetrical and conserved structure of the biotinoyl domain of the BCCP subunit is disrupted by a structured loop called the “thumb” that protrudes from body of the domain. Prior structural work showed that the thumb interacts with *uriedo* ring of the attached biotin moiety. We have tested whether the thumb-biotin interactions are responsible for the greater holo form stability by examination of two BCCP-87 species that lack the thumb. These BCCP species were produced in both the apo and holo forms, and their sensitivities to trypsin digestion were compared. The holo forms of these proteins were found to be only marginally more stable than their apo forms and much more sensitive to trypsin digestion than the wild type holo-BCCP-87. Therefore, removal of the thumb has an effect similar to lack of biotinylatation, indicating that thumb-biotin interactions are responsible for most (but not all) of the increased stability of the holo biotinoyl domain. In the course of these experiments we demonstrated that treatment of *Escherichia coli* with the peptide deformylase inhibitor, actinonin, results in the expected (but previously unreported) accumulation of an N-formylated protein species.

1 The acetyl-CoA carboxylase (ACC)1 (EC 6.4.1.2) of *Escherichia coli* is a multisubunit enzyme belonging to the biotin carboxylase family, a class of enzymes that use biotin to transfer an activated carboxyl group from the carboxylation site to a second site where carboxyl transfer occurs. The biotin moiety is covalently attached to a specific lysine residue in the biotin carrier domain of the carboxylase (1). In *E. coli* ACC the biotin carrier function resides on a 156-residue protein, the biotin carboxyl carrier protein (BCCP), of which the C-terminal half comprises the biotin carrier domain (1, 2). The biotin group is attached post-translationally to a lysine residue from the C terminus by the enzyme biotin ligase, which in *E. coli* is the multifunctional BirA protein that is also the transcriptional repressor of the biotin biosynthetic operon (3).

The first form of *E. coli* BCCP isolated was a 9.1-kDa biotin carrier protein active in the carboxylation reaction (4) that was subsequently shown to be a stable proteolytic fragment of the intact subunit. A very similar protein was produced by digestion of BCCP with subtilisin Carlsberg (5, 6). Subsequent *in vivo* experiments showed that both the subtilisin fragment (BCCP-80), and the 9.1-kDa protein (BCCP-82), comprising the C-terminal 80 and 82 residues, respectively, contain all the sequences information necessary for proper folding of the biotinoyl domain and hence for biotinylatation (7, 8). BCCP-82 was used to obtain the three-dimensional structure of the biotinylated (holo) form of the biotinoyl domain of *E. coli* BCCP by x-ray crystallography (5), whereas the C-terminal 87 residues of BCCP (BCCP-87) was used for multidimensional nuclear magnetic resonance (NMR) (9–11) analyses. The two methods gave very similar structures for the holo domain. (The differing N termini of the proteins studied are of no consequence, since both N termini are highly mobile and thus were not observed. Only the last 77 residues of BCCP are ordered). The protein forms a flattened β-barrel structure with the biotinoyl-lysine exposed on a tight β-turn composed of the conserved Ala-Met-Lys-Met biotinylatation motif. The BCCP biotin domain adopts a fold strikingly similar to those of several domains modified by lipoyl acid attachment (12). The structure of the unbiotinylated (apo) form of the BCCP-87 biotinoyl domain determined by NMR (9, 10) is very similar to that of the holoprotein. Both forms of the protein have the same basic fold, although there are reported differences in the more defined structures.

The biotinylated lysine residue of the BCCP biotin domain is at the tip of a protruding hairpin turn (5, 9, 11), and thus modification of this residue would not be expected to affect the overall structure or dynamics of the protein. However, we pre-

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1 The abbreviations used are: ACC, acetyl-CoA carboxylase; BCCP, biotin carboxyl carrier protein; Tricine, N-(2-hydroxy-1,1-bis(hydroxy methyl)ethyl)glycine.
RESULTS

Biotin Attachment by Chemical Acylation Is Sufficient to Stabilize the BCCP Biotinoyl Domain—Our first hypothesis (13) concerning the stabilization of the BCCP biotinoyl domain was that the increased stability may not due to biotinylation per se, but rather could be a consequence of interactions with the BirA biotin ligase, the enzyme catalyzing the modification reaction. The rationale for this hypothesis was that the reaction mechanism of E. coli biotin ligase is known to involve major structural rearrangements of the enzyme (21–23), and it seemed possible that these conformational changes could be relayed to the biotinoyl domain substrate. Moreover, alterations in domain tertiary structure upon biotinylation could aid in dissociation of the ligase-holo biotinoyl domain complex. These putative ligase-domain interactions would result in changing the conformation of the domain such that it became more stably folded than the apo form. Subsequent determinations of the structure of the apo-protein that allowed comparison with the holo form showed that conformational changes would necessarily be small (9–11). However, there are several reported differences in the structures of the holo and apo domains that could have been imparted by interaction with BirA during the biotinylation reaction. For example, Yao and co-workers (10) reported that the hairpin turn that contains the biotinylated lysine is twisted in the holo form, but is not twisted in the apo form.

The hypothesis of a conformational change imparted by interaction with BirA was difficult to test, because biotin ligase treatment was the only method available to specifically biotinylate the domain, and we know of no method to remove the biotin moiety from the intact domain. We now report a means to test this hypothesis by preparation of a biotin domain modified such that the lysine residue targeted for biotinylation was the sole amino group of the protein. This allowed biotinylation by use of a chemical acylation reaction that was specific to amino groups under the experimental conditions chosen.

The “sole-amino” form of the biotin domain was prepared in two stages. In the first stage all of the lysine residues except that targeted for biotinylation were converted to arginine residues by oligonucleotide cassette mutagenesis of the BCCP-87 coding sequence (17). Arginine was chosen to retain the sensitivity of the protein to trypsin digestion, since this is a valuable conformational probe of BCCP-87 structure (13, 19). The substitutions of arginine for lysine also retained the charge and extended the aliphatic chain of the original residues. As expected from the surface location of the lysine residues, the modified protein (called 4K to 4R) seemed well folded, since it was readily purified by our standard protocol and was a good substrate for biotin ligase in vitro and in vivo. More definitive was the observation that the 4K to 4R domain retained full function in vivo when converted to a full-length BCCP (17).

Although arginine residues cannot be chemically acylated at physiological pH values (due to the very high $pK_a$ values of guanidino groups) (24), the 4K to 4R domain retained a group in addition to that of the target lysine that could be acylated, the N terminus of the protein. This amino group cannot be removed by mutagenesis, and since N termini generally have lower $pK_a$ values than lysine e-amino groups (24), selective acylation of the target lysine was precluded. Therefore, we explored several in vivo modifications to block the BCCP N terminus and found N-formylation to be the most effective means. We took advantage of the pathway of initiation of protein synthesis in bacteria (25) in which the N-terminal methionine is incorporated as the N-formylated species by use of the initiator tRNA carrying N-formylmethionine. In the normal course of protein synthesis the N-formyl group is cleaved.
from the nascent peptide by peptide deformylase. Following deformylation the methionine residue is removed by methionine aminopeptidase, but only if the penultimate residue has a small side chain (25). Our prior work showed that methionine aminopeptidase is inactive on formyl peptides, and thus methionine removal is dependent on deformylase action (16). We also showed that an N-formylated biotinyl domain accumulated in vivo when peptide deformylase activity was blocked by mutation (16). However since peptide deformylase is an essential enzyme, synthesis of the N-formylated protein required several cumbersome manipulations. Moreover, these manipulations generated large amounts of a yellow compound (presumably a folate metabolite) that complicated purification of the domain (16). Since that work was reported, actinonin, a broad-spectrum antibiotic of previously unknown function, has been shown to be a specific inhibitor of the E. coli peptide deformylase targeted to the active site (26). Actinonin addition has no effect on wild type E. coli strains, because the efflux pump rapidly exports the inhibitor. However when the pump is inactivated by mutation of the acr or tolC genes, actinonin becomes a potent inhibitor of growth (26). Although accumulation of N-formylated proteins had not been demonstrated in actinonin-treated E. coli cells, the finding that growth inhibition was overcome by overproduction of the deformylase indicated that the primary target of actinonin was peptide deformylase (26).

To use actinonin as a peptide deformylase inhibitor we inactivated the acr efflux pump by introduction of a tolC::Tn10 mutation into the host strains used for protein expression. By judicious addition of cultures to cultures of the tolC strain induced for expression of the 4K to 4R BCCP-87 domain we obtained large amounts of the N-formylated apo species of the protein that was readily purified to homogeneity by ion exchange chromatography and had a mass of 9474.1, a value in excellent agreement with the calculated mass of 9473.9 (Fig. 1A). (This to our knowledge is the first demonstration of the accumulation of an N-formylated protein due to actinonin treatment.) It should be noted that we were unable to completely inhibit deformylase action even at very high actinonin concentrations and thus had to separate the N-formyl apo-BCCP-87 species from the other BCCP-87 species. A possible explanation for the partial inhibition observed is that peptide deformylase can use a variety of metal ions that impart greatly differing actinonin sensitivities (26). It seems possible that in vivo a mixture of different metal ion forms of peptide deformylase is present. As expected the N-formyl apo-BCCP-87 had a trypsin sensitivity quite similar to that of the unformylated protein. Indeed by reversed phase high pressure chromatography all of the BCCP species examined in this work were found to release marker trypsic peptides in the same pattern and relative amounts as previously demonstrated for the wild type and several mutant proteins (13, 16, 19) (data not shown). We then used biotinyl-N-hydroxysuccinimide to biotinylate the sole amino group of the N-formyl apo-BCCP-87 protein, that of the lysine 122 side chain. Nondenaturing gel electrophoresis showed that the chemical modification procedure shifted the N-formyl apo-BCCP species to an electrophoretic mobility identical to that of the protein biotinylated in vivo, indicating that a positive charge had been neutralized and mass spectral analysis (Fig. 1B) gave a mass of 9699.8 for the chemically biotinylated protein, a value in excellent agreement with the calculated mass of 9700.2. These results showed that each biotinylated N-formyl BCCP molecule contained only a single amide-linked biotin moiety, thereby demonstrating the specificity of the acylation reaction. It should be noted that BCCP-87 contains a single cysteine residue that upon reaction with biotinyl-N-hydroxysuccinimide would give a biotin thioester. However, cysteine biotinylation was not observed presumably due to the inaccessibility of the cysteine residue (which resides in the hydrophobic core of the domain) plus the conditions used for the acylation reaction (which were conducive to thioester hydrolysis).

We then compared the trypsin sensitivity of the chemically biotinylated protein to that of biotinylated protein obtained by biotinylation in vivo (Fig. 2, A and B). Several sites in BCCP-87 have very similar sensitivities to trypsin cleavage in both the apo and holo species (13, 16, 19) such that the digestion appears as an all-or-none reaction on denaturating gel electrophoresis (13), and thus the only bands present on these gels are those of the full-length proteins. Therefore, quantitation of the rate of digestion by loss of band intensity was straightforward. The trypsin sensitivities of the two proteins were very similar, but more importantly upon chemical biotinylation the N-formyl 4K to 4R protein became as stable as the holo form obtained by
biotinylation in vivo. In both cases the holo forms were only slightly digested upon 21 h of trypsin treatment, whereas the apoproteins were largely or completely digested. Interpretation of these results was straightforward; interaction of the biotin domain with the BirA biotin ligase was not required to obtain a protein having the reduced trypsin sensitivity of the holo domain. Chemical biotinylation was sufficient to stabilize the apo protein toward trypsin digestion (see below) and a better substrate for in vitro biotinylation by BirA, although the two proteins showed comparable biotinylation in vivo (17).

The two thumbless proteins were produced in both the apo and holo forms, purified to homogeneity, and their relative trypsin sensitivities tested (Fig. 2, C and D). We found that the apo forms of both thumb deletion proteins were slightly more sensitive to trypsin digestion than the 4K to 4R background in this laboratory (17). In the Reche/Perham thumbless construct (called BCCP-Th (P)) the thumb was cleanly excised, whereas in our construct (called BCCP-Th (C)) the thumb was replaced by a single alanine residue inserted to avoid a putative unfavorable Ramachandran angle predicted by molecular modeling. We found the two proteins to behave similarly except that BCCP-Th (P) was the more stable protein toward trypsin digestion (see below) and a better substrate for in vitro biotinylation by BirA, although the two proteins showed comparable biotinylation in vivo (17).

We have approached this question by examining the comparative stabilities of the apo and holo forms of two proteins missing the thumb structure. If the stabilization of the biotin domain is due to biotin-thumb interactions, deletion of the thumb should mimic the absence of biotinylation. That is, biotinylation should do little or nothing to stabilize the thumbless BCCPs, and therefore, the apo and holo forms of the “thumbless” proteins would show similar sensitivities to trypsin digestion.

One of these thumbless BCCP-87 species was constructed from the wild type gene during the ligase specificity studies of Reche and Perham (27), whereas the second was constructed in the 4K to 4R background in this laboratory (17). In the Reche/Perham thumbless construct (called BCCP-Th (P)) the thumb was cleanly excised, whereas in our construct (called BCCP-Th (C)) the thumb was replaced by a single alanine residue inserted to avoid a putative unfavorable Ramachandran angle predicted by molecular modeling. We found the two proteins to behave similarly except that BCCP-Th (P) was the more stable protein toward trypsin digestion (see below) and a better substrate for in vitro biotinylation by BirA, although the two proteins showed comparable biotinylation in vivo (17).

Fig. 2. Protease sensitivities of BCCP-87 species. In each panel the black bars represent the holo form, and the gray bars represent the apo form of each protein examined. The amount of intact protein was measured by denaturing gel analysis (see “Experimental Procedures”). The BCCP species examined were all BCCP-87 derivatives (thumbless proteins have less than 87 residues). The holoproteins were obtained either by in vivo biotinylation or by treatment of the apoprotein with the BirA biotin ligase except in B where the apoprotein was modified by treatment with biotinoyl-N-hydroxysuccinimide. A, BCCP 4K-4R; B, N-formyl-BCCP 4K-4R; C, BCCP-Th(C); and D, BCCP-Th(P). There was no detectable BCCP-87 remaining at the 21-h time point of B.

Stabilization of Biotinoyl Domain of E. coli Acetyl-CoA Carboxylase

Our previously reported data demonstrated that the holo-BCCP-87 domain is considerably more stable toward proteolysis and cysteine modification than the holo form. These data could be explained by the apo and holo forms having different structures or differing dynamics (or both). Since that report two groups have examined the structures of the apo and holo forms of BCCP-87 by NMR techniques (9, 10). Although there are some disagreements between the two studies, both report that the apo and holo biotinoyl domains have the same overall structure. Therefore, our prior data must indicate that the apo form has a more dynamic structure than the holo form. This is reasonable since the chemical and proteolytic probes we used were irreversible and thus well suited to detection of altered dynamics (28). Altered dynamics were also shown by one of the NMR studies that specifically compared the final energies of the structural ensembles of the apo and holo forms and found that final energy of the holo form ensemble to be ~507 kJ/mol, whereas that of the apo-BCCP-87 ensemble was significantly greater (~241 kJ/mol) (9). Another NMR study showed that the order parameters calculated from 15N-relaxation times of the protein backbone were generally smaller for the apo form, thus indicating a lower degree of stability, although no values for the overall stabilities of the apo and holo forms were given (11).
Our chemical biotinylation results indicate that the differences between the holo and apo form are due solely to the presence of the covalently attached biotin. These data specifically exclude models in which interaction with biotin ligase converts BCCP-87 from one conformer to another of similar or lower overall energy. Therefore, the observed stabilization of the biotin domain structure conferred by biotinylation must be understood in terms of the biotin-protein interactions. In the first structure of the BCCP biotin domain, the crystal structure of Athappilly and Hendrickson (5), the two hydrogen bonds were reported to link atoms of the biotin uriedo ring and Thr-94 of the protruding thumb structure. Specifically, the Thr-94 side chain and main chain oxygen atoms formed hydrogen bonds with the carbonyl and N1'-H atoms of the biotin uriedo ring, respectively. There were a priori reasons to doubt the biological relevance of the reported bonds. The angle of the hydrogen atom in the first of these reported bonds was only 111°, the bond was rather long (3.8 Å), and Thr-94 is not a conserved residue in other BCCPs, some of which are known to function

FIG. 3. Diagrams of the effects of biotinylation and removal of the thumb. The left figure is that of holo-BCCP-87 with the biotin moiety in an interacting position; the central figure is that of apo-BCCP-87 with the lysine side chain in the same rotamer conformation as that of the left figure; and the right figure is a generic model of the thumb deletion forms of holo-BCCP-87 with the biotin moiety in the same position as that of the left figure.

This data, several weak nuclear Overhauser interactions are found between the protons of the biotin moiety and protons of most of the thumb residues. The proton chemical shifts of the thumb residues also differ in the apo and holo forms. Finally, the dynamics of the thumb residues differ in the two forms in that the thumb of the apoprotein is more mobile than the thumb of the holo form (9, 11). Therefore, although it is clear that the biotin rings and the thumb interact, the interactions cannot be restricted to a specific geometry since virtually all of the thumb residues show interaction with biotin (9, 11). Biotin is too small to interact with all of these residues simultaneously unless the thumb structure collapses around the biotin moiety. However, the structure of the thumb is the same in the apo and holo forms, so collapse is excluded. Finally, residues distant from the thumb show weak interactions with biotin ring protons showing that the biotin rings can access regions distant from the thumb region (9) and therefore cannot be tightly bound in place. It should be noted that early circular dichroism data indicated that the biotin moiety of the holo biotinoyl domain interacted with the body of the protein (30).

We are therefore left with a dynamic picture in which the biotin rings have restricted mobility, but are not frozen in place. Our analyses of the thumbless biotinoyl domain proteins indicate that most, but not all, of the observed stabilization is due to biotin-thumb interactions that both slow the dynamics of the thumb (11) and prejudice the location of the biotin rings. The interactions seem to be heterogeneous within the population, such that in one molecule the biotin uriedo ring may be interacting with Tyr-92, whereas in a neighboring BCCP-87 molecule the interaction may be with Thr-94, Ser-92, or a nontooth residue. What we lack are values for the strengths of the various biotin-thumb interactions and whether or not these interactions are sufficiently large to give the observed greater stability of the holo form (9). NMR studies of a thumbless BCCP domain would be very useful in this regard.

Since the thumb is required for the acetyl-CoA carboxylase reaction (17), the biotin-thumb interactions may have a physiological role. If the thumb requirement is due to the interactions of this protein segment with the biotin moiety, then the reason may be an example of the “hot potato” hypothesis of Perham (31) in which the carboxylated form of the biotin moiety must interact with the thumb to stabilize the unstable enzyme intermediate until it can be delivered to the active site where it is consumed. However, carboxylbiotin has a long half-life (>100 min at pH 8) (32), and other biotinylated proteins lack the thumb (5, 17), and thus the hot potato hypothesis seems unlikely to apply in this case. Another hypothesis seems more attractive. In this hypothesis the biotin-thumb interac-
ations serve to align the biotin moiety for accesses to the ACC active sites. In this scenario the diversity of biotin-thumb interactions might suggest that different biotin-thumb alignments are required to access the biotin carboxylase and carboxyltransferase active sites. These aligning interactions would result in decreased mobility of both partners giving the observed greater stability of the holo form of the BCCP biotioyl domain. Since the thumb structure is thus far found only in the biotinoyl proteins of bacterial and chloroplast ACCs (17), it seems that in other biotinylated enzymes, the postulated align-

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