Covalent Modification of an Exposed Surface Turn Alters the Global Conformation of the Biotin Carrier Domain of *Escherichia coli* Acetyl-CoA Carboxylase*

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We have studied the apo (unbiotinylated) and holo (biotinylated) forms of BCCP87, an 87-residue COOH-terminal peptide comprising the biotin carrier domain of the biotin carboxyl carrier protein subunit of *Escherichia coli* acetyl-CoA carboxylase. The apo protein spontaneously formed disulfide-linked dimers and was modified readily by sulfhydryl reagents, whereas the holo protein remained monomeric and was unreactive toward sulfhydryl reagents unless a protein denaturant was present. These data indicated that the single cysteine residue of the domain (Cys-116) was much more reactive in the apo form of the protein. Incubation of apoBCCP87 with biotin ligase for different times, followed by reaction with fluorescein-5-maleimide, clearly showed that the loss of Cys-116 reactivity was the result of modification with biotin. In addition, reaction of Cys-116 with 5,5'-dithiobis(2-nitrobenzoic acid) showed that apoBCCP87 denatured at lower urea concentrations than holoBCCP87. We also found that apoBCCP87 was at least 10-fold more sensitive than the holo form to proteolysis by a range of proteases. Identification of the cleavage sites indicated that the differences in protease sensitivity could not be attributed to shielding of susceptible bonds by the biotin moiety of the holo protein. These data indicate that a conformational change accompanies biotinylation of the biotin domain. Thus, modification of a β-turn protruding from the protein surface results in alteration of the overall structure of this protein domain.

Acetyl-CoA carboxylase (EC 6.4.1.2) from *Escherichia coli* is a multi-subunit enzyme belonging to the biotin carboxylase family, enzymes that use a biotin moiety 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 acetyl-CoA carboxylase, the biotin carrier function is located in a 156-residue protein, the biotin carboxyl carrier protein (BCCP),² of which the COOH-terminal half comprises the biotin carrier domain (2, 3). The biotin group is attached post-translationally to a lysine 34 residues from the COOH terminus (1) 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 (4).

Early work on purification of BCCP from *E. coli* produced a 9.1-kDa biotin carrier protein active in the carboxylation reaction (5). This protein was shown subsequently to be a stable proteolytic fragment of the intact subunit and to be very similar to the protease-resistant, biotin-containing fragment produced by digestion of BCCP with subtilisin Carlsberg (6, 7). Subsequent in *vivo* experiments showed that both the subtilisin fragment (BCCP80) and the 9.1-kDa protein (BCCP82), comprising the COOH-terminal 80 and 82 residues, respectively, contain all the sequence information necessary to specify biotinylation (8, 9).

Biotin domain proteins share considerable sequence homology with the functionally analogous lipoyl attachment regions of pyruvate dehydrogenase (10). The recently solved crystal structure of biotinylated (holo) BCCP80 (11) shows that the domain adopts the same basic fold as several lipoyl domains (12–14), as predicted from the sequence similarities between the two protein families (15). Dardel et al. (16) concluded from the NMR spectra of lipoylated and unlipoylated forms of the domain that there is little conformational change associated with lipoylation.

Both BCCP82 and intact BCCP purified from *E. coli* contain a single cysteine residue (Cys-116 of the intact protein) that is inaccessible to sulfhydryl reagents in the native state (5, 6). This finding is consistent with the orientation of Cys-116 in the crystal structure of the holo form of BCCP80 (11), where the residue side chain is buried and forms part of the hydrophobic core. We have previously reported the expression and purification of both the apo (unbiotinylated) and holo forms of a slightly larger 87-residue COOH-terminal biotin domain peptide from BCCP (17). A recent kinetic analysis of BCCP87 shows that interaction of BCCP87 and the intact BCCP with biotin ligase are essentially identical (18). Here, we present data showing that, unlike post-translational modification of the similar lipoyl domain, biotinylation of BCCP87 is accompanied by a conformational change resulting in alterations in the reactivity of Cys-116 and the susceptibility of the domain to a variety of proteases.

**EXPERIMENTAL PROCEDURES**

**Protein Methods**—PAGE and detection of biotinylated proteins was performed as described previously (17). Band intensities were quantitated with a Molecular Dynamics Computing Densitometer 300A, using ImageQuant software.

Expression, purification, and quantitation of apo- and holoBCCP87 were carried out as described previously (17). Preparations of each form...
Peptides were recovered by SpeedVac concentration for analysis by 0–50% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v) over 50 min. Reaction with Sulfhydryl Reagents—apo and holoBCCP87 were lyophilized and redissolved in 50 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was then added to produce the final concentrations given in the figure legends, and the reaction followed at 412 nm in a Shimadzu UV-160A spectrophotometer at 30 °C. Modification under denaturing conditions was performed by dissolving protein samples in 50 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, which either contained urea (SigmaUltra) at the concentration indicated or in 50% AcN. Where necessary for UV spectroscopy, minor contamination with small nucleic acids present after Q-Sepharose chromatography was eliminated by gel filtration.

Protein samples (10–50 μg) were treated with fluorescein-5-maleimide (FM; Pierce) in 40 mM sodium phosphate (pH 6.5), at 25 °C, or [125I]iodoacetamide (IAM; Amersham, specific activity 17.6 mCi/mmol) in 50–100 mM Tris-Cl (pH 9.0) at 37 °C, with a 5-fold molar excess of FM or IAM over cysteine residues. The reaction was quenched by addition of a 20-fold molar excess of β-mercaptoethanol.

In Vitro Biotinylation—ApoBCCP87 (50 μM) was incubated at 37 °C with 1.25 mM E. coli biotin ligase (the kind gift of Dr. D. Beckett, University of Maryland Baltimore County, Baltimore, MD) in the presence of 3 mM ATP, 5.5 mM MgCl2, 60 mM biotin, 100 mM KCl, and 5 mM dithiothreitol in 20 mM sodium phosphate (pH 7.0). Samples were removed into EDTA (final concentration 20 mM) to stop the reaction. Protease Digestion and Peptide Identification—Protein (0.35–0.5 mg/ml) was treated with chymotrypsin or trypsin (Boehringer Mannheim, sequencing grade), or subtilisin Carlsberg (Sigma) at 25 °C in 100 mM Tris-Cl (pH 7.9) containing 50 mM NaCl and 10 mM CaCl2. Digestion with endoproteinase Arg-C (Boehringer Mannheim, sequencing grade) or subtilisin Carlsberg (Sigma) was carried out at a protein concentration of 0.5 mg/ml according to the manufacturer. Reactions were stopped by snap freezing at −80 °C and addition of EDTA to a final concentration of 20 mM, where appropriate. To prevent further proteolysis, gel samples were thawed in the presence of SDS/β-mercaptoethanol gel loading buffer at 100 °C for 5 min immediately before loading, and samples for HPLC were thawed in the presence of 0.1% trifluoroacetic acid. Digestion products were separated by reverse phase HPLC on a C4 column (Brownlee Aquapore, 1.7-μm particle size, 2.1 × 100 mm) essentially as described by Hobba et al. (19) with elution over a linear gradient of 0–50% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v) over 50 min. Peptides were recovered by SpeedVac concentration for analysis by HPLC, terminal sequencing and mass spectrometry as described previously (17).

Fluorescence and UV Absorbance Spectroscopy—Intrinsic tyrosine fluorescence data was acquired and analyzed in an Aminco Bowman Series 2 Luminescence Spectrometer using an excitation wavelength of 280 nm and collecting data at 0.2-nm intervals with five repetitions. The sample temperature was maintained at 25 °C. UV spectra were measured in a Varian Cary 3 spectrophotometer and the second derivative spectra calculated from the data using an in-house program based on the method of Savitzky and Golay (20).

RESULTS

Our first indication that the cysteine residue of apoBCCP87 was more reactive than that of holoBCCP87 was the detection of disulfide dimers in some preparations of the apo protein by electrospray mass spectrometry (data not shown) and SDS-PAGE (Fig. 1, lane 4). In contrast, we found only traces of dimer in our holoBCCP87 preparations, consistent with the reported inaccessibility of the cysteine thiol group (Fig. 1, lane 3; the faint band in this lane with mobility of the disulfide-linked dimer was <1% of the total protein, and most probably represents dimer formed from denatured protein during sample preparation or electrophoresis). The apoBCCP87 dimers were readily reduced by β-mercaptoethanol to give the monomeric species (Fig. 1, lane 2). Incubation of the holo protein with SDS prior to SDS-PAGE resulted in conversion of disulfide-linked holoBCCP87 dimers (dimer became 12% of total protein) and an increase in apo protein dimers to around 20% (Fig. 1, lanes 5 and 6). Dimer formation during storage was a relatively slow process, with 9% of apoBCCP87 present as dimers after 4 months at −20 °C.

Sulfhydryl Modification—In agreement with the early work on BCCP82 and intact BCCP (5, 6), holoBCCP87 reacted with DTNB only in the presence of SDS or urea (Fig. 2). Only traces of a DTNB reaction with native holoBCCP87 was detected in a 17-h incubation (data not shown). In contrast, native apoBCCP87 gave a readily detected rate of modification (Fig. 2), which resulted in quantitative reaction of the thiol group within several hours (data not shown). The rates of modification of both forms of BCCP87 were increased in the presence of SDS and markedly increased on addition of urea (Fig. 2). However, the difference observed in reactivity of the two species persisted in the presence of the denaturants, where with either SDS or urea, the apo protein reacted more rapidly than the holo protein. Two other sulfhydryl reagents, FM (Fig. 3) and [14C]I (data not shown), gave very similar results. When samples of apo and holo protein were incubated with either of these reagents followed by analysis on PAGE, incorporation of 14C or fluorescent material into apoBCCP87 was linear with time for at least 2 h. No incorporation into the holo protein was detected over this time interval (data not shown). The differing reactivities of Cys-116 in the apo and holo forms indicated that it should be possible to follow the progress of the biotin ligase reaction by the loss of reactivity of the cysteine thiol. When freshly reduced apoBCCP87 was incubated with biotin ligase, biotin, and ATP, the total amount of FM-reactive protein decreased progressively with time until no reaction with the reagent could be observed (Fig. 3) and, as expected from the prior data, FM labeled only the apo form.

The observation that the increased reactivity of apo protein over holo protein persisted in the presence of urea and SDS (Fig. 2) suggested that apoBCCP87 may be more readily denatured. Therefore, we used the reactivity of Cys-116 toward DTNB to follow unfolding of the two proteins. Samples of apo- and holoBCCP87 were incubated in varying concentrations of urea, before determining the initial rate of modification by DTNB. Expressing these rates as a proportion of the rate in 8 M urea (Fig. 4) generated curves which showed that apoBCCP87 unfolded at a lower concentration of urea than holoBCCP87, with half-maximal modification occurring at urea concentrations of 4.3 M and 5.2 M, respectively.

Proteolysis—Since the cysteine modification and denaturation data suggested a conformational difference upon biotinylation, we investigated the susceptibility of the two forms of the protein to a range of proteases having different specificities. Initially, the proteins were treated with chymotrypsin, trypsin or subtilisin Carlsberg as described under “Experimental Procedures,” and samples of the digest taken over time were analyzed on SDS-PAGE (Fig. 5). The results show that apoBCCP87...
was considerably more susceptible to all three proteases, with decreasing quantities of the intact 10-kDa protein detected over 4 h and none remaining after 21 h of incubation. In contrast, quantitation of the bands by laser densitometry indicated that 85–90% of the holo protein was intact after 21 h of digestion (data not shown). As expected from the data of Fall and Vagelos (6, 7), subtilisin Carlsberg trimmed the amino terminus of holoBCCP87 to produce species of slightly greater electrophoretic mobility (presumably holoBCCP80) but was otherwise protease-resistant. Although the sequence of BCCP suggested that some peptide fragments of intermediate size might be produced, the initial cleavage products appeared to be rapidly degraded to fragments that were too small for analysis by SDS-PAGE.

One possible explanation for the observed difference in the protease digestion patterns was direct protection of susceptible residues by the biotin moiety of the holo protein. Therefore, we have used limited proteolysis and peptide mapping to determine where the proteins were first cleaved by trypsin and endoproteinase Arg-C. Digestion of apoBCCP87 with Arg-C released two peptides, which were detected in the chromatograms at the earliest time point (Fig. 6b). The peptides were identified as A2 and A1 (Fig. 6c) by mass spectrometry (Table I). A peak corresponding to the remaining portion of the protein was not observed on HPLC, presumably due to the generation of an insoluble core peptide as seen by Sutton et al. (2) in trypsin digests of holoBCCP80. In contrast, there was no initial Arg-C digestion of holoBCCP87, but the same two peptides were released when the incubation times were extended (Fig. 6c). A third major peak, A3, was subsequently released in both apo and holo protein samples. This was identified as peptide T8 (Table I), and is apparently due to a contaminating trypsin-like activity in the Arg-C preparation. When apoBCCP87 was subjected to digestion with trypsin (Fig. 6d), the peptides that were first released at low levels corresponded to T1, T2, and T8 (Table I), i.e. we did not detect an initial cleavage at Lys-122, which is the biotinylated residue of the holo protein. We failed to recover material corresponding to the undigested portion of the molecule as expected (2). Cleavage at Lys-122 was evident after 15 min digestion at a protease:BCCP ratio of 1:100 (w/w). The holo protein was not digested by trypsin under these conditions (Fig. 6e); however, after 60 min of exposure at a protease:BCCP ratio of 1:40 (w/w), peptides T1, T2, T8, and minor amounts of T4 were released (data not shown). Similar digests with chymotrypsin gave at least eight peaks detected by HPLC after 15 min of digestion of apoBCCP87 at a protease: BCCP ratio of 1:200 (w/w), and several of these peaks were shown to contain multiple species when analyzed by mass spectrometry (data not shown). This is consistent with the observed absence of large peptide products (Fig. 5). Although the complexity of the chymotrypsin digestion products precluded further analysis, the HPLC profiles of apo- and holoBCCP87 confirmed the differing susceptibility seen on SDS-PAGE.

Intrinsic Tyrosine Fluorescence and UV Spectra—BCCP87 contains a single tyrosine residue, and comparison of the fluorescence emission spectra produced upon excitation of the two forms of BCCP87 at 280 nm revealed a 2-fold higher relative fluorescence in the holo protein, with no change in the emission maximum (Fig. 7a). The quantum yield was not significantly affected in the presence of 0.15 M NaCl (reduced by 7% and 3% in apo and holo forms, respectively). Second derivative UV
absorption spectra indicated a red shift in the maxima from the apo to holo state in the wavelengths contributed by the tyrosine residue. The observed $l_{\text{max}}$ and $l_{\text{max}}$ shifted from 284.25 nm and 276.8 nm, respectively, in the apo protein to 285.5 nm and 278.2 nm after biotinylation (Fig. 7b). There was no alteration to the phenylalanine region of the spectrum. These differences were independent of salt and protein concentration (data not shown).

**DISCUSSION**

The observed difference in the ability of the two forms of BCCP87 to form disulfide-linked dimers (Fig. 1) was consistent with the reactivity of Cys-116 toward sulfhydryl reagents. Comparison of the modification of apo- and holoBCCP87 with DTNB (Fig. 2), FM and IAN indicated that whereas Cys-116 was readily accessible in apoBCCP87, it was much less available for reaction in holoBCCP87. The latter result is consistent both with previous modification studies (5, 6) and the x-ray crystal structure of holoBCCP80 (11). The crystal structure also indicates that the lack of reactivity of Cys-116 in the native holo protein is not due to steric shielding by the biotin moiety. The only residue masked by the biotin ring structures is Leu-117, where the solvent accessible surface area is decreased by about half (11). The Cys-116 side chain is located on the other side of the $\beta$ strand, and thus is well removed and blocked from interaction with the biotin moiety (Fig. 8). Furthermore, we have shown that the side chain of Cys-116 became inaccessible to modification as biotinylation proceeded (Fig. 3). The denaturation curves of the proteins (Fig. 4) indicated that apoBCCP87 had a more open structure which unfolded at a lower urea concentration than the holo protein. These data are consistent with a conformational change in the region around Cys-116 accompanying biotinylation.

The considerably slower rate of proteolytic cleavage by Arg-C and trypsin in holoBCCP87 that we have observed suggests that the conformational change extends to other regions of the molecule. This interpretation is consistent with the study of Fontana et al. (21), who demonstrated with apo- and holomyoglobin that limited proteolysis is a reliable method for detecting structural differences. The change in protease susceptibility of BCCP87 was not due to the physical proximity of the biotin

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**TABLE I**

Analysis of peptides produced by digestion of BCCP87 with endoproteinase Arg-C and trypsin

| Peptide identity | Retention time | Mass predicted | Mass determined |
|-----------------|---------------|----------------|-----------------|
| A1              | 23.98         | 1551.8         | 1551.4          |
| A2              | 21.04         | 1057.2         | 1056.8          |
| A×              | 34.38         | 2183.5         | 2206.8          |
| T1              | 23.83         | 1551.8         | 1551.8          |
| T2              | 20.95         | 1057.2         | 1056.7          |
| T3              | Not detected  | 714.8          |                 |
| T4              | 18.22         | 891.0          | 891.6           |
| T5              | 30.94         | 1491.8         | 1491.8          |
| T6 + T7         | 17.08         | 1551.8         | 1551.8          |
| T8              | 34.07         | 2183.5         | 2183.5          |

*Refer to Fig. 6a.*

*The additional mass of 23 units is most probably due to the presence of a tightly bound sodium ion (17).*

*Peptides were identified by NH$_2$-terminal sequencing.*

*Not detected in digests of holoBCCP87.*

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**FIG. 6.** Limited proteolysis of BCCP87 analyzed by reverse phase HPLC. a, sequence of BCCP87 showing the predicted peptides from cleavage with Arg-C (A) and trypsin (T). Residues are numbered with respect to the sequence of intact BCCP, and the site of attachment of biotin (Lys-122) is indicated. b and c, Arg-C; apo (b) and holo (c) BCCP87 were digested with Arg-C at a protease:BCCP ratio of 1:100 (w/w) for varying times and the products separated by HPLC as described under “Experimental Procedures.” The chromatograms represent digestion for 60 min, with the inset showing peak areas for the major products over the time course, calculated using Waters Maxima software. d and e, trypsin; apo (d) and holo (e) BCCP87 were digested with trypsin at a protease:BCCP ratio of 1:200 (w/w) for 15 min and analyzed by HPLC.
moiety, since Arg-93 is 11 Å away from the biotin and Arg-84 and Lys-136 are on the opposite face of the molecule (Fig. 8). In fact, while there was a difference in the rate of proteolysis, it was clear that the same residues were the most susceptible in both forms of the protein. Thus it appears that, despite the location of Lys-122 in the apo protein, both forms of the protein. Thus it appears that, aside from the site of attachment, the biotin moiety of holoBCCP is free to interact with the biotin-binding proteins, avidin and streptavidin (3, 17). Our favored interpretation of the present data is that biotinylation of BCCP87 results in a marked stabilization of the protein, resulting in a less dynamic structure that is reflected in the decreased reactivity of the cysteine residue and increased protease resistance. Thus, titration of the cysteine residue of the apo form would result from the increased kinetic availability of the cysteine side chain plus the irreversible nature of the modification reactions. Structural stabilization of proteins by substrates and other ligands is well known. However, in most instances, the sites of ligand binding are found within the protein structure where the ligand forms numerous interactions that stabilize the protein. Good examples are the stabilization of streptavidin by biotin binding (24) and the stabilization of myoglobin by heme binding (21). In the crystal structure of holoBCCP80 (11), only two interactions (other than modification of Lys-122) were observed between the biotin moiety and the protein. The side chain and main chain oxygen atoms of Thr-94 are hydrogen-bonded to the biotin ureido carbonyl (O2') and N1' atoms, respectively. However, neither of these interactions seems likely to play important roles in either BCCP structure or ligase interaction. Thr-94 is part of a protruding thumb structure that is missing in several otherwise well conserved biotin domains (11), which are known to be effective substrates for E. coli biotin ligase (8, 25). Moreover, even in those biotin domains that contain a putative thumb structure, a threonine residue is not conserved at this position. Finally, the observed Thr-94-ureido hydrogen bonds seem unlikely to be physiologically relevant. N1' of biotin is the site of carboxylation during acetyl-CoA carboxylase action, and thus the hydrogen bond could not persist during enzyme function. Furthermore, the angle of the hydrogen bond to the Thr-94 side chain oxygen atom is a highly unusual 111°, suggesting that the interaction may result from crystal packing constraints. Indeed, Roberts (26) in NMR investigations of the solution structure of holoBCCP87 found no evidence of hydrogen bonding between the ureido ring and Thr-94, and recent NMR exchange studies of holoBCCP87 failed to detect any hydrogen bonds between the biotin moiety and the protein.2

Thus it appears that (aside from the site of attachment) the biotin moiety has little direct interaction with the protein mol-

2 R. W. Broadhurst, personal communication.
ecule and cannot directly stabilize the domain structure. Therefore, by default we attribute the BCCP87 conformational change to binding and release of the biotin domain by biotin ligase. We propose that a conformational change within the biotin ligase acts to convert BCCP87 from the cysteine-accessible, protease-susceptible conformer to the cysteine-buried, protease-resistant conformer. Biotin ligase is known to have at least three conformational states depending on which ligands are bound (27). These conformational states can differ markedly in thermodynamic stability and may involve rather large structural rearrangements. For example biotin ligase crystals shatter upon addition of biotin or biotinoyl-AMP (28). Therefore, BirA conformational changes may be sufficient to alter the conformation of BCCP87. A plausible rationale for the BCCP conformational change would be to release the biotinylated domain from biotin ligase. It remains to be seen whether such conformational changes are a common feature of biotin carrier proteins.

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