Acetyl Coenzyme A Carboxylase

PROTEOLYTIC MODIFICATION OF BIOTIN CARBOXYL CARRIER PROTEIN*

(Received for publication, September 14, 1972)

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SUMMARY

The biotin carboxyl carrier protein (BCCP) component of Escherichia coli acetyl coenzyme A carboxylase has been previously isolated in multiple active forms, ranging in molecular weight from 45,000 to 9,100. Since the apparent native form of BCCP is the largest of these species, the isolation of the small forms was attributed to proteolysis during the purification procedures employed. We have now shown that native BCCP is very susceptible to subtilisin hydrolysis. Limited proteolysis of crude or purified preparations of native BCCP with subtilisin Carlsberg produces a mixture of BCCP species analogous to those previously noted. After more extensive proteolysis with subtilisin Carlsberg, all the intermediate forms are converted to a small form of BCCP, termed BCCPc, which we have isolated in milligram quantities and compared with BCCPc, the smallest form of BCCP previously isolated (mol wt 9100). Purified BCCPc was found to be homogeneous by electrophoresis in polyacrylamide gels in the presence or absence of sodium dodecyl sulfate, and in both gel systems it was indistinguishable from BCCPc, indicating the similarity in size and charge between the two proteins. From isoelectric focusing in polyacrylamide gels, an isoelectric point of approximately 4.5 was determined for both BCCPc and BCCPc. The amino acid composition of BCCPc (80 amino acid residues, including 1 half-cystine residue) was identical with that of BCCPc, except that BCCPc contains 2 additional alanine residues. The single sulfhydryl residue in either protein was titratable with 5,5'-dithiobis-(2-nitrobenzoic acid) only when denaturants such as 8 M urea or 1% sodium dodecyl sulfate were added. The biotin content of both proteins is consistent with the presence of 1 biotin residue per molecule. BCCPc was active as a carboxyl acceptor and donor in the biotin carboxylase and transcarboxylase reactions of acetyl-CoA carboxylase. In each of these reactions a $K_m$ of approximately $3 \times 10^{-5}$ M was determined for BCCPc compared with a value of $2.5 \times 10^{-3}$ M for BCCPc. $V_{max}$ values for the two proteins in these reactions were essentially identical.

When BCCPc (96 amino acid residues; mol wt 10400), previously isolated along with BCCPc from a mixture of small BCCP species, was treated with subtilisin Carlsberg, it was quantitatively hydrolyzed to a species of BCCP identical with BCCPc. BCCPc and BCCPc are resistant to further proteolysis at 37°C and pH 8 by high concentrations of subtilisin as well as trypsin, chymotrypsin, and thermolysin, lending support to the idea that these small forms represent a stable "core" peptide in native BCCP. Efforts to isolate the peptide fragment lacking biotin from subtilisin-treated BCCP have been unsuccessful, suggesting that it is rapidly degraded by subtilisin.

The acetyl coenzyme A carboxylase of Escherichia coli has been resolved into three functionally distinct protein components, including biotin carboxyl carrier protein, biotin carboxylase, and transcarboxylase (1, 2). BCCP plays a central role as the carrier for an activated carboxyl group as indicated in Scheme 1. The biotin prosthetic group of BCCP is carboxylated in a Mn$^{2+}$ and ATP-dependent reaction catalyzed by the biotin carboxylase component to form CO$_2$-BCCP (2, 3). The third protein component, the transcarboxylase, catalyzes the transfer of the carboxyl group from CO$_2$-BCCP to acetyl-CoA, forming

![Scheme 1](https://example.com/scheme1.png)

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* This investigation was supported in part by Grant GB-5142X from the National Science Foundation and Grant 1-R01-HL10406 from the National Institutes of Health.

† Recipient of National Heart and Lung Institute Postdoctoral Fellowship 5-P49-HL-10609-07.
malonyl-CoA (4, 5); this reaction regenerates BCCP, which is then available to accept another carboxyl group.

Several species of BCCP, ranging in molecular weight from 49,000 to 9,100 and varying in activity in the biotin carboxylase and transcarboxylase reactions, have been isolated from E. coli extracts (6–9). The resolution of these forms by polyacrylamide gel electrophoresis is represented diagrammatically in Fig. 1. The initial attempts to purify large quantities of BCCP resulted in a preparation which contained multiple forms (Fig. 1, mix). Each BCCP in this mixture was purified to homogeneity and the preparation which contained multiple forms (Fig. 1, mix). Each BCCP contained multiple forms (Fig. 1, mix). Each BCCP was purified to homogeneity and the preparation which contained multiple forms (Fig. 1, mix). Each BCCP in this mixture was purified to homogeneity and the isolated fractions were characterized. All four fractions were equally active in the reconstituted acetyl-CoA carboxylase reaction, exhibiting $K_m$ values of approximately 2.5 $\times 10^{-3}$ M as well as similar $V_{\text{max}}$ values (9). The fastest migrating form (Fraction 1 in Fig. 1) has been isolated in the largest quantities and crystallized (6). It contains 82 amino acid residues and 1 biotin residue, giving a residue molecular weight of 9,084 (this form of BCCP is referred to as BCCP(9100), for which the subscript denotes the approximate molecular weight). Fraction 2 BCCP contains approximately 96 amino acid residues and 1 biotin residue with a residue molecular weight of 10,408 (this form of BCCP is referred to as BCCP(9100o)). Fractions 3 and 4 were less well characterized but appeared to be very similar to BCCP(9100). The existence of these multiple forms of BCCP suggested that some limited proteolysis had occurred during the isolation procedure.

Recently we have presented evidence to suggest that the apparent native form of BCCP in E. coli is a much larger and more active protein than any of the previously isolated small forms (7, 8). The isolated apparent native form of BCCP (referred to hereafter as simply BCCP) is shown in Fig. 1 as the slowest migrating BCCP band. Purified BCCP has a molecular weight of 45,000, and it contains two similar polypeptide chains of molecular weight 22,500. Each polypeptide chain contains approximately 207 amino acid residues and 1 residue of biotin (8). The purified BCCP exhibits $K_m$ values in the biotin carboxylase and transcarboxylase reactions of approximately $2 \times 10^{-3}$ M and $4 \times 10^{-3}$ M, respectively, which are 50- to 100-fold lower than $K_m$ values for any of the small forms. All the purified forms of BCCP give similar $V_{\text{max}}$ values in these reactions.

A comparison of the amino acid composition of BCCP with those of BCCP(9100) and BCCP(9100o) supported the suggestion that these smaller forms were derived from BCCP by proteolysis. However, we were unable to repeat the isolation of any of the small forms of BCCP or to obtain any evidence for the proteolysis of BCCP to these small forms in crude extracts (8). We concluded that proteolysis must have occurred in association with the special handling procedures used during the large scale purification of the small forms of BCCP (6).

This communication presents direct evidence for the production of active, small forms of BCCP by proteolytic modification of native BCCP. In addition, the properties of a purified, subtilisin Carlsberg-modified BCCP (termed BCCP(9100)) are presented and compared with those of BCCP(9100).

**EXPERIMENTAL PROCEDURE**

**Materials**

The sources or the procedure for the preparation of most of the reagents and enzymes used in this study have been previously described (8). In addition DNase and RNase (both protease-free), chymotrypsin, trypsin, subtilisin Carlsberg, and subtilisin BPN' were obtained from Sigma. a-Aminopeptidase was obtained from P-L Biochemicals. Thermolysin and carboxypeptidases A and B were gifts of Dr. Ralph Bradshaw. $\nu$-[3H]Biotin was prepared by catalytic exchange (New England Nuclear) and found to be approximately 40% radiochemically pure. Therefore, it was purified essentially as described by Ogata (10). A solution of the impure $\nu$-[3H]biotin in methanol (22 mg, 660 mCi) was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 ml of 5 m imidazole-chloride, pH 7.0, and applied to a column (0.9 x 15 cm) of Dowex 1-X2-formate (200 to 400 mesh). The column was washed with water until no further radioactivity was detected in the effluent and then eluted with 0.012 M formic acid at a flow rate of 2 ml per min; 5- to 6-ml fractions were collected and analyzed for radioactivity by liquid scintillation counting of 1-ml aliquots in Bray's solution (11). Numerous peaks of radioactivity were detected and each was analyzed by thin layer chromatography (12) and bioassay (using Lactobacillus plantarum (13)) for $\nu$-biotin. The $\nu$-[3H]biotin peak eluted between 500 and 600 ml and was pooled and lyophilized. The dry residue was dissolved in 5 m imidazole-chloride, pH 7.0, and rechromatographed exactly as above. The center of resulting peak of $\nu$-[3H]biotin was pooled and lyophilized. The final residue was dissolved in 20 ml of methanol and stored at −15°C. The purified $\nu$-[3H]biotin was greater than 98% pure as measured by thin layer chromatography on Silica Gel G plates developed with chloroform-methanol-acetic acid (70:20:3:5) ($R_F$ biotin = 0.5 (12)). The over-all yield of $\nu$-[3H]biotin was 30%, and the specific activity of the final preparation was determined to be 2.6 Ci per mmole by bioassay (13).

For some experiments $\nu$-[3H]biotin-labeled cells of E. coli PA-502 were used; this biotin auxotroph was a gift of Dr. F. Jacob, and its growth in the presence of $\nu$-[3H]biotin has been previously described (2, 8). For most of the experiments reported $\nu$-[3H]biotin-labeled E. coli strain S was used. This strain, a gift of Dr. E. C. Liu, was grown to 5% log phase at 37°C in Vogel and Bonner medium E (14) containing 0.5% glucose and 20 μg per liter of $\nu$-[3H]biotin (2.6 Ci per mmole). This level of biotin is sufficient to repress biotin synthesis (15) and allows incorporation of $\nu$-[3H]biotin into BCCP in wild type strains at levels comparable with the initial steps in the large scale purification of BCCP(9100) and BCCP(9100o) were carried out at the Enzyme Center at Tufts University, and it is difficult to assess the exact conditions employed.
those obtained with biotin auxotrophs. E. coli K-12 cells were obtained from Grain Processing Corp., Muscatine, Iowa. Lactobacillus plantarum 8014 was obtained from the American Type Culture Collection.

**METHODS**

**Enzyme Assays**—Acetyl-CoA carboxylase was assayed spectrophotometrically under conditions of limiting biotin carboxylase or limiting transcarboxylase as previously described (6, 7).

**Analytical Polyacrylamide Gel Electrophoresis**—Electrophoresis of BCCP preparations was carried out in 10% acrylamide gels (0.3% methylenebisacrylamide) using the pH 8.9 system of Davis (13) as previously described (8). This type of gel will be referred to as a standard pH 8.9 gel. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using the method of Weber and Osborn (17). [biotin-3H]BCCP on polyacrylamide gels was detected as previously described (8), by slicing frozen gels and determining the radioactivity in individual gel slices.

**Proteolysis of [biotin-3H]BCCP Preparations**—For most of the proteolysis studies described preparations of [biotin-3H]BCCP were incubated with various proteases, and then the [biotin-3H]-protein products were analyzed by polyacrylamide gel electrophoresis. Routine incubation mixtures intended for analysis by standard pH 8.9 gels contained 10 to 200 µg of protein (10,000 to 30,000 dpm of various [biotin-3H]BCCP preparations), 0.01 M Tris-Cl, pH 8.0, and H2O to a total volume of 0.04 ml. Reactions were started by addition of 0.01 ml of freshly prepared solutions of proteases in 0.01 M Tris-Cl, pH 8.0. After incubation at 37° for the appropriate times (usually 10 min) the reactions were terminated by rapid freezing in Dry Ice-acetone. Special precautions were taken to avoid continued proteolysis during the handling necessary to apply samples to the gels. Frozen samples were thawed one by one by the addition of an equal volume of cold 40% glycerol (containing a trace of bromphenol blue to serve as tracking dye), and rapidly applied to the surface of gels (cooled to 4°) with disposable micropipettes. Electrophoresis was carried out at 4°. As an added precaution in studies of subtilisin digestion, the reactions were terminated by the addition of 2 ml phenylmethane sulfonyl fluoride (from a 0.2 M solution in 1-propanol) just prior to freezing. Incubation mixtures intended for sodium dodecyl sulfate gel electrophoresis (subtilisin-treated samples) had a total volume of 0.025 ml. Proteolysis was terminated by addition of phenylmethane sulfonyl fluoride (as above) followed by 0.003 ml of 10% sodium dodecyl sulfate, and the mixture was placed in a boiling water bath for 2 min. The warm samples were adjusted to 1% 2-mercaptoethanol, 0.005 ml of 50% glycerol (containing a trace of tracking dye) was added, and the samples were layered onto sodium dodecyl sulfate gels.

Various crude and purified preparations of [biotin-3H]BCCP were subjected to proteolysis treatment. The purified preparations have been previously described (8) and are indicated in the text. Most studies with crude preparations utilized E. coli strain 8 cells which had been grown in the presence of [3H]biotin (see "Materials"). The cells were harvested at 3/4 log phase and washed twice with either 0.02 M Tris-Cl, pH 8.0, or 0.02 M sodium phosphate, pH 7.0. Crude extracts of these cells were prepared by suspending the cells in a small volume of the same wash buffer and passing the cold suspension through a French pressure cell at 20,000 p.s.i. The homogenate was centrifuged at 48,000 × g for 30 min at 4°, and the supernatant fraction served as the crude extract. Typical extracts contained 20 to 25 mg of protein per ml and approximately 200,000 dpm of [3H]-biotin per mg.

**Miscellaneous**—The following determinations were performed as previously described in detail (8): protein by a microbiuret procedure (18); biotin contents of [biotin-3H]BCCP preparations by Pronase digestion followed by an avidin binding assay (19, 20), or by stoichiometric carboxylation with 44Clbicarbonate (1); sulfhydryl titrations of BCCP preparations with 5,5'-dithiobis-(2-nitrobenzoic acid) (21, 22); amino acid analyses of purified BCCP preparations by the general methods of Moore and Stein (23), Hirs (24), and Edelhoch (25); molecular weight estimations on a calibrated Sephadex G-100 column (26); isoelectric points by isoelectric focusing in polyacrylamide gels essentially as described by Rigby and Drysdale (27) except that 7.5% acrylamide, 0.2% methylenebisacrylamide gels were used; and sedimentation coefficients by sedimentation velocity measurements.

**Isolation of BCCP(80)** Studies described under the "Results" suggested that a small form of BCCP (referred to as BCCP(80)), which was very similar in size to BCCP(9100), could be produced in high yields from partially purified preparations of BCCP which were treated with subtilisin Carlsberg. A typical purification of [biotin-3H]BCCP from such a preparation of [biotin-3H]BCCP is described below.

**Hydrolysis of [biotin-3H]BCCP and Ammonium Sulfate Fractionation**—[biotin-3H]BCCP was purified through Step 3 as previously described (8) from 1 pound of E. coli K-12 cells harvested at 3/4 log phase (Grain Processing Corp.) supplemented with 10 g of [3H]biotin-labeled cells of E. coli strain 8 (grown as described in "Materials"). The ammonium sulfate precipitate from Step 3 was dissolved in 0.02 M Tris-Cl, pH 8.0, and adjusted to contain a protein concentration of 20 mg per ml, 2.5 mM MgCl2, 25 µg per ml each of DNase and RNase (protease free) and 1 mg of subtilisin Carlsberg per 300 mg of protein. The mixture was incubated at 37° for 10 min, then adjusted to contain 2 mM phenylmethane sulfonyl fluoride, and after 1 min, ammonium sulfate was added to 45%, saturation (0.258 g per ml). After stirring for 15 min at 4° the precipitate was collected by centrifugation at 27,000 × g for 15 min.

**Sephadex G-100 Chromatography**—The precipitate from the above step was dissolved in 8 ml of 0.02 M potassium phosphate, pH 7.0, 5% glycerol, and applied to a column (2.5 × 80 cm) of Sephadex G-100 equilibrated with the same buffer (minus glycerol). The flow rate was approximately 20 ml per hour, and 3-ml fractions were collected. Fractions were analyzed for protein and radioactivity as previously described (8). The elution profile is shown in Fig. 2. The fractions eluting between 275 and 325 ml were pooled and concentrated by pressure filtration (UM-2 membrane, Amicon Corporation) to less than 5 ml and stored at −15°.

**Preparative Polyacrylamide Gel Electrophoresis**—The pool from above was adjusted to 5% glycerol, a drop of bromphenol blue was added, and the mixture was applied to a preparative polyacrylamide gel containing a 24-ml separating gel (15% acrylamide, 0.15% methylenebisacrylamide) and a 4-ml stacking gel, which was prepared and run as previously described (8, 28). The elution buffer flow rate was 1 ml per min and 3-ml fractions were collected. The elution profile is shown in Fig. 3. The [biotin-3H]BCCP(80) in fractions 17 to 26 was shown to give a single radioactive protein band on analytical gels, and these fractions were pooled and lyophilized. The dry residue was dis-

1 R. R. Fall and P. R. Vagelos, unpublished observations.
BCCP(,,,. A sample of [biotin-3H]BCCP(,,, (285 mg of protein, 10.7 x 10^6 dpm) was applied to the column and eluted as described in the text. Fractions (3 ml) were analyzed for protein (absorbance at 280 nm) and radioactivity as described in the text.

solved in a minimal volume of 0.01 M potassium phosphate, pH 7.0, and this solution was dialyzed exhaustively against the same buffer at 4°C. The dialyzed preparation of [biotin-3H]BCCP(60) was stored at -15°C. The results of a typical purification procedure are shown in Table I.

RESULTS

Degradation of BCCP in Crude Extracts

Initial Attempts to Reisolate BCCP(4500) and BCCP(10400)—In a previous report of the isolation of BCCP (8) we attempted to reproduce the experimental conditions which gave rise to BCCP(4500), BCCP(10400), and other small forms of BCCP (6, 9). The molecular forms of [3H]biotin-labeled BCCP in crude extracts were monitored by gel filtration on Sephadex G-100. In those studies (8) with extracts of E. coli PA-502, a biotin auxotroph, we failed to observe the production of any small forms of BCCP corresponding to BCCP(9000) or BCCP(16400), even when crude extracts were incubated for 4 hours at 37°C under apparently identical conditions to those described earlier (e.g. extracts prepared in 0.05 M Tris-Cl, pH 8.0, and supplemented with RNase and DNase at 25 μg per ml). The only observed effect on BCCP was an apparent dissociation (possibly coupled with limited proteolysis) of BCCP dimer (mol wt 45,000) to a monomeric form (approximate mol wt 22,500). Since commercially grown E. coli B cells (Grain Processing Corp.) were used for the isolation of the small forms of BCCP, we tested the possibility that the PA-502 cells (an E. coli K-12 derivative) may lack the appropriate protease (or proteases) necessary for the hydrolysis of BCCP by adding crude extracts of E. coli B cells to PA-502 extracts. Again, an examination of these extracts after incubation for 4 hours at 37°C by gel filtration failed to reveal any forms of BCCP corresponding to a molecular weight less than 20,000. In addition, we fractionated a mixture of E. coli B and PA-502 cells through the first three steps of the purification procedure used for the isolation of BCCP(4500) (6), thinking that proteolysis may occur at some later stage of the procedure. Here too, no forms of BCCP corresponding to BCCP(9000) or BCCP(10400) were detected.

Since the isolation of BCCP(9000) and BCCP(16400) could not be repeated, we considered various possible explanations: (a) the batch of commercially obtained cells used for the isolation of the small forms of BCCP may have contained a source of protease not normally associated with E. coli cells; (b) the special problems of handling large quantities of protein may have allowed conditions for the endogenous proteases present in E. coli (29) to hydrolyze BCCP; and (c) a proteolytic enzyme may have been unintentionally introduced during the purification procedure, possibly in the RNase and/or DNase which were added to the crude extract. The addition of subtilisin Carlsberg6 on the molecular

4 In recent experiments extracts of [3H]biotin-labeled E. coli B were noted to produce a [biotin-3H]-peptide of approximately 15,000 daltons; we have not yet further characterized this species.

5 With one preparation of DNase tested, some proteolysis of the biotin-protein was noted to occur through the first three steps of the purification procedure used for the isolation of BCCP(4500) (6), thinking that proteolysis may occur at some later stage of the procedure. Here too, no forms of BCCP corresponding to BCCP(9000) or BCCP(10400) were detected.

6 Two commercial preparations of subtilisin were used in this study, corresponding to subtilisin Carlsberg and subtilisin BPN' as described by Ottesen and Svendsen (35).
forms of BCCP in a crude extract is illustrated in Fig. 4. Crude extracts of [3H]biotin-labeled E. coli PA 502 were prepared as previously described (8) and incubated at 37°C for 1 hour in the presence or absence of 1 part subtilisin Carlsberg per 1,000 parts protein. The elution profiles for the [3H]biotin-labeled proteins in such extracts are shown in Curves 1 to 5; Curve 4 represents the elution profile of untreated [3H]BCCP (dimer, mol wt 45,000). When a similar extract was heated at 37°C for 1 hour, essentially all of the dimer was converted to a monomer (mol wt 22,500; Curve 2). These results are identical with those previously presented (8). When the extract was heated in the presence of subtilisin Carlsberg, essentially quantitative conversion to a low molecular weight peak of [3H]protein was noted (Curve 5) coincident with the elution position of authentic [biotin-3H]-BCCP (9100) (Curve 4). These results were the first clear indication of a proteolytic process which could cause the conversion of BCCP to a polypeptide chain of approximately 10,000 daltons. This prompted us to investigate in detail the effects and products of subtilisin treatment of BCCP, as well as the general effects of other proteases on the hydrolysis of BCCP in crude extracts.

Polyacrylamide Gel Electrophoresis Experiments—In order to follow the proteolytic modification of BCCP preparations it was advantageous to use analytical polyacrylamide gel electrophoresis to evaluate the products formed, since multiple forms of BCCP are readily resolved in 10 or 15% gels (pH 8.9 system of Davis (16)) as illustrated in Fig. 1. Preparations of [biotin-3H]BCCP were treated with various proteases and then electrophoresed on gels, followed by staining and determination of the radioactivity in individual gel slices (see “Experimental Procedure” for details and precautions used to avoid further proteolysis during the handling of samples). Gels were not fixed in trichloroacetic acid after electrophoresis but were rapidly frozen to avoid loss of trichloroacetic acid-soluble radioactivity.

Some examples of the use of this procedure to study the proteolysis of [biotin-3H]BCCP preparations are illustrated in Fig. 5. For these experiments the source of [biotin-3H]BCCP was a crude extract of E. coli strain 8 which had been grown on minimal media supplemented with [3H]biotin as described under “Experimental Procedure.” This strain of E. coli was chosen for these studies because it shows less tendency than several other strains tested for the dissociation of BCCP to a monomer in crude extracts.3 Fig. 5A shows the radioactivity profile of a sliced 10% gel containing crude extract incubated at 37°C for 10 min (O---O); superimposed is the profile of a similar gel containing [biotin-3H]BCCP (9100) (O---O). The major radioactive peak (O---O) corresponds to BCCP (native dimer) and the minor peak (O---O) represents the amount of BCCP monomer.

Fig. 5. Polyacrylamide gel electrophoresis of a crude preparation of [biotin-3H]BCCP treated with various proteases. The details of the incubation of a [3H]biotin-labeled crude extract of E. coli strain 8 with various proteases and the subsequent analysis of radioactive products by electrophoresis in 10% acrylamide gels (pH 8.9) is described in the text. A, a sample of extract (100 µg of protein, 19,800 dpm) warmed to 37°C for 10 min at pH 8 (O---O), and a sample of untreated [biotin-3H]BCCP (9100) (O---O). B, a sample of the extract as in A treated with 1 µg of thermolysin (O---O) or 1 µg of subtilisin Carlsberg (O---O). C, a sample of the extract as in A treated with 1 µg of trypsin (O---O) or 1 µg of subtilisin BPN’ (O---O). D, a sample as in A, treated with 1 µg of chymotrypsin (O---O) or 1 µg of Pronase (O---O). On the right side of the figure the mobilities of BCCP and BCCP (9100) are indicated for reference purposes. Open circles which coincided with closed circles are not indicated. The last slice represents the tracking dye front.
formed in 10 min at 37°. Fig. 5, B, C, and D, illustrate the types of [biotin-3H]peptide products formed by incubation of the same extract for 10 min at 37° with various proteases added in a ratio of 1:100 parts of protease to parts of total protein in the extract. Fig. 5B shows the pattern of [biotin-3H]peptides produced when either subtilisin Carlsberg (O--O) or thermolysin (●--●) were added to the extract. Fig. 5C shows the pattern when subtilisin BPN' (O--O) or trypsin (●--●) were added. Fig. 5D shows the pattern resulting from the addition of Pronase (O--O) or chymotrypsin (●--●). At the concentration tested, all of the proteases except trypsin caused essentially complete hydrolysis of BCCP to a variety of [biotin-3H]peptides. Material migrating as rapidly as BCCP(9100) was detected after treatment with both subtilisins (Carlsberg and BPN') and Pronase. No in case was there a large amount of [3H]biotin-labeled material at the tracking dye front. When shorter incubation times were used, or lower levels of proteases were added, patterns of multiple [biotin-3H]peptide products (not shown) were seen with all the proteases tested, except for trypsin which had no apparent effect on BCCP in crude extracts. Some of the data obtained from these experiments is summarized in Table II. Various levels of the listed proteases were added to crude extracts for 10 min at 37°, and the resulting hydrolysis of BCCP and production of [biotin-3H]peptides co-migrating with BCCP(9000) were determined. There was no simple way to tabulate the other multiple peaks of [biotin-3H]peptides, so they are not included. The proteases are listed in the order of effectiveness for hydrolyzing BCCP in crude extracts. The most effective proteases tested were the subtilisins which hydrolyzed greater than 80% of the BCCP in 10 min at pH 8 and 37° at a protease to protein ratio of 1:10,000. Pronase and thermolysin were somewhat less effective at this concentration, hydrolyzing approximately 50% of the BCCP. Chymotrypsin was less active than the above, whereas trypsin was completely ineffective as mentioned. Other proteases without apparent effect under these conditions (data not shown) were α-amino- and carboxypeptidases A and B.

Of the proteases tested, only the subtilisins and Pronase produced [biotin-3H]peptide material with the same electrophoretic mobility as BCCP(9000); subtilisin Carlsberg was the most effective in this regard, producing essentially quantitative conversion to a small biotin peptide (hereafter referred to as BCCP(990)) at a ratio of 1:100. Subtilisin BPN' consistently produced a mixture of material similar to BCCP(900) and a slower migrating species (as seen in Fig. 5C). The production of the rapidly migrating [biotin-3H]peptide by Pronase may be due to the presence of a subtilisin-type protease in this commercial protease preparation; a recent report has shown that commercial Pronase contains many exo- and endopeptidases, one of which is similar to the subtilisins (35).

Since BCCP(9000) is the best characterized of the small forms of BCCP previously isolated, and the subtilisins produced a similar biotin peptide in high yields from BCCP, further studies described here will be concerned with the proteolytic modification of BCCP by subtilisin. The products of hydrolysis of BCCP by chymotrypsin, Pronase and thermolysin have not been characterized further, but clearly they may represent active BCCP species worthy of further investigation.

Production of [biotin-3H]Peptides by Subtilisin Hydrolysis of [biotin-3H]BCCP—The time course of the subtilisin catalyzed hydrolysis of crude [biotin-3H]BCCP was examined by standard and sodium dodecyl sulfate polyacrylamide gel electrophoresis in order to determine both the pattern and approximate polypeptide chain molecular weight of the resulting [biotin-3H]peptides. In a typical experiment a crude preparation of [biotin-3H]BCCP was subjected to hydrolysis with subtilisin Carlsberg at 37° and pH 8, using a protease to protein ratio of 1:300. After 1, 2, 4, and 8 min of proteolysis, 2 mM PMSF was added to terminate the reaction. Each hydrolysate was then analyzed on standard and sodium dodecyl sulfate gels. The resulting distribution of radioactivity between [biotin-3H]BCCP and [biotin-3H]peptides as determined from the sliced gels is shown in Fig. 6; Fig. 6, A and B, represent the standard gels and Fig. 6, C and D, represent the sodium dodecyl sulfate gels. The migration positions of BCCP and BCCP(9000) are indicated on the figures; other molecular weight markers are also indicated in Fig. 6, C and D. Standard gels (Fig. 6, A and B) illustrate a complex pattern of intermediate [biotin-3H]peptides at short times (1, 2, and 4 min) and the conversion of all of these forms to BCCP(900) at 8 min. Sodium dodecyl sulfate gels of these same hydrolysates (Fig. 6, C and D) reveal a much simpler pattern involving the appearance with time of one asymmetrical peak of [biotin-3H]peptide (or peptides) coupled with the disappearance of [biotin-3H]BCCP. At short times the distribution of radioactivity in the [biotin-3H]peptide peak appeared to be skewed more towards the top of the gel.

Table II

<table>
<thead>
<tr>
<th>Ratio of protease to protein</th>
<th>BCCP hydrolyzed</th>
<th>Apparent BCCP(9100) produced†</th>
<th>Recovery of radioactivity§</th>
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<td></td>
<td>%</td>
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† Identified by electrophoresis at Rf 0.8 on standard 10% gels.
‡ In each case essentially all the radioactivity entered the gel.
§ A small peak at Rf 0.54 represented apparent dissociation of BCCP to a monomeric form in 10 min at 37°; values are corrected for 11% dissociation.
towards a polypeptide chain molecular weight between 10,000 and 12,000, while at 8 min the peak is shifted to coincide with BCCP(9100). These results taken together suggest that the multiple \([\text{biotin-3H}]\)peptide peaks seen on standard gels correspond to peptides of very similar size (mol wt 9,000 to 11,000); at early times the peptides are distributed towards the higher molecular weight (perhaps corresponding to BCCP(18000)), whereas, at later times, only the smallest peptide, BCCP(9100), accumulates. Clearly, during the subtilisin hydrolysis of BCCP there is no accumulation of intermediate size biotin peptide chains (between 22,500 and 11,000 daltons), suggesting that the subtilisin cleavage of BCCP may involve the hydrolysis of only one or two "sensitive" bands which results in the formation of a biotin-containing fragment and a fragment containing no biotin. The biotin-containing fragment appears to be further hydrolyzed to a "stable core" peptide represented by BCCP(9100). The multiple \([\text{biotin-3H}]\)peptides detected on standard gels at short times suggest that the previous isolation of a mixture of BCCP fragments (Reference 9 and as shown in Fig. 1, mix) may have been the result of limited proteolysis of BCCP by a subtilisin-type protease.

**Subtilisin Treatment of Purified Preparations of BCCP**

Since the treatment of crude extracts with subtilisin appeared to produce small BCCP species very similar to those previously isolated (6, 9), it was of interest to examine more closely the effect of subtilisin on purified or partially purified preparations of BCCP in order to attempt to isolate milligram quantities of these small forms. 

**Polyacrylamide Gel Electrophoresis of Subtilisin-treated BCCP**—An apparently homogeneous preparation of \([\text{biotin-3H}]\)BCCP (isolated as described previously (8)) was subjected to treatment with subtilisin Carlsberg and subtilisin BPN' under conditions similar to those described for crude extracts. Stained 10% standard polyacrylamide gels of BCCP alone and after treatment with subtilisin Carlsberg or subtilisin BPN' in ratios of 1:300 to 1:30,000 parts of protease to parts of BCCP (wt/wt) for 10 min at 37°C and pH 8 are shown in Fig. 7. Gel 1 shows 30 µg of untreated \([\text{biotin-3H}]\)BCCP (the smearing is characteristic of BCCP and has been previously noted (8)). Gels 2, 3, and 4 show the effects of treatment of a similar amount of BCCP with 1, 10, and 100 ng of subtilisin Carlsberg, respectively. With low levels of subtilisin Carlsberg, multiple protein bands are seen, whereas, at the highest level (100 ng), only one band is detectable. This rapidly migrating protein which has been termed BCCP(9100), migrates as rapidly as BCCP(9100). Gel 5). When these gels were sliced and the radioactive bands determined, the resulting profiles were essentially the same as noted with crude extracts (Fig 6, A and B). Thus, these results show visually the same course of proteolysis that was noted with proteolysis in crude extracts. Several efforts were made to detect a nonradioactive peptide on gels that would correspond to the fragment of BCCP that contains no biotin, if indeed subtilisin splits the protein into two fragments. Only on Gel 3 was a major nonradioactive protein band detected (corresponding to the second fastest migrating band), and, therefore, this might be the fragment lacking biotin or a derivative thereof. However, this band does not accumulate under any of the conditions tested, suggesting that it is rapidly degraded by subtilisin. We considered the possibility that the fragment lacking biotin may not be resolved from the biotin-containing fragment under these conditions of electrophoresis, but this seems unlikely in light of the results described below. Gels 6 and 7 show the effect of 1 and 100 ng of subtilisin BPN', respectively, on 30 µg of BCCP. The results are similar to those obtained with subtilisin Carlsberg, except that two radioactive protein bands were always noted at high levels of protease (Gel 7). Thus, these results show visually the same course of proteolysis that was noted with proteolysis in crude extracts. Several efforts were made to detect a nonradioactive peptide on gels that would correspond to the fragment of BCCP that contains no biotin, if indeed subtilisin splits the protein into two fragments. Only on Gel 3 was a major nonradioactive protein band detected (corresponding to the second fastest migrating band), and, therefore, this might be the fragment lacking biotin or a derivative thereof. However, this band does not accumulate under any of the conditions tested, suggesting that it is rapidly degraded by subtilisin. We considered the possibility that the fragment lacking biotin may not be resolved from the biotin-containing fragment under these conditions of electrophoresis, but this seems unlikely in light of the results described below. Gels 6 and 7 show the effect of 1 and 100 ng of subtilisin BPN', respectively, on 30 µg of BCCP. The results are similar to those obtained with subtilisin Carlsberg, except that two radioactive protein bands were always noted at high levels of protease (Gel 7). The faster migrating band is essentially identical with BCCP(9100) and BCCP(9100). The slower band probably differs from BCCP(9100) by only a few amino acid residues. Since it was invariably present when BCCP preparations were treated with subtilisin BPN', this protease was not used in the isolation of a small BCCP similar to BCCP(9100), because these two proteins proved difficult to resolve from one another on a preparative scale.

Instead, subtilisin Carlsberg was used to prepare BCCP(9100) which can easily be obtained in homogeneous form (as described below and under "Experimental Procedure"). When a known inhibitor of the subtilisins, phenylmethane sulfonyl fluoride (36), was incubated at a concentration of 2 mM for 1 min with 50 ng of subtilisin Carlsberg and 50 ng of subtilisin BPN' prior to the addition of 30 µg of BCCP, little apparent proteolysis occurred in 10 min (Gel 8), although some minor bands appeared which are not clearly visible in the photograph. Phenylmethane sulfonyl fluoride (2 mM) was used routinely to terminate the subtilisin mediated hydrolysis of BCCP.
FIG. 7 (left). Polyacrylamide gel electrophoresis of purified BCCP treated with subtilisin. Standard 10% acrylamide gels (pH 8.9) were run as described in the text. Gel 1, 30 μg of untreated [biotin-3H]BCCP. Gel 2, 30 μg of [biotin-3H]BCCP treated with 1 ng of subtilisin Carlsberg for 10 min at 37° and pH 8. Gel 3, as in Gel 2 except with 10 ng subtilisin Carlsberg. Gel 4, as in Gel 2 except with 100 ng of subtilisin Carlsberg. Gel 5, 10 μg of authentic BCCP(9300). Gel 6, as in Gel 2 except with 1 ng of subtilisin BPN'. Gel 7, as in Gel 2 except with 100 ng of subtilisin BPN'. Gel 8, as in Gel 2 but with 2 mM phenylmethane sulfonyl fluoride prior to addition of 50 ng each of subtilisin Carlsberg and BPN'. None of the stained bands represents subtilisin Carlsberg or BPN' which did not enter the gel. The direction of electrophoresis was from top to bottom, as shown.

FIG. 8 (center). Polyacrylamide gel electrophoresis of BCCP(9100) and BCCP(9100) treated with subtilisin. Standard 10% acrylamide gels (pH 8.9) were run as described in the text. Gel 1, 10 μg of BCCP(9100). Gel 2, 10 μg of BCCP(9100) treated with 10 μg of subtilisin Carlsberg at 37° for 10 min at pH 8. Gel 3, 15 μg of BCCP(9100). Gel 4, 15 μg of BCCP(9100) treated as in Gel 2 with 5 ng of subtilisin Carlsberg. Gel 5, as in Gel 4 but treated with 500 ng of subtilisin Carlsberg. None of the stained bands represent subtilisin Carlsberg which did not enter the gel. The direction of electrophoresis was from top to bottom, as shown.

FIG. 9 (right). Isoelectric focusing of BCCP(9100) and BCCP(9100) in polyacrylamide gels. Samples of BCCP(9100) and BCCP(9100) were focused in pH 3 to 6 gradients as described in “Experimental Procedure.” The top of the gels represents pH 6 and the bottom, pH 3. Gel 1, 10 μg of BCCP(9100) prepared as described in this communication. Gel 2, 10 μg of BCCP(9100). Gel 3, 15 μg of a second preparation of BCCP(9100).

Stability of BCCP(9100) and BCCP(9100) to Proteolytic Digestion—The fact that subtilisin Carlsberg treatment of BCCP leads to the accumulation of a small stable biotin-containing fragment, BCCP(9100), and the apparent disappearance of the fragment lacking biotin suggests that BCCP(9100) represents a resistant “core” portion of the native BCCP. To further examine this point, the stability of BCCP(9100) as well as BCCP(9100) to further hydrolysis by high concentrations of subtilisin Carlsberg was tested. In addition samples of BCCP(9100) were treated with subtilisin Carlsberg to test the premise that it represents an intermediate stage of proteolysis between BCCP and BCCP(9100). The results of one such experiment are shown in Fig. 8, where samples of BCCP(9100) and BCCP(9100) are shown on 10% standard polyacrylamide gels before and after treatment with subtilisin Carlsberg. Not shown are gels of subtilisin Carlsberg alone (10 μg) which were free of detectable protein bands because the protein did not pass through the stacking gel. Gel 1 shows 10 μg of untreated BCCP(9100) and Gel 2 shows 10 μg of BCCP(9100) which has been treated with 10 μg of subtilisin Carlsberg for 10 min at 37° and pH 8. Even at this level of subtilisin, which is 1000-fold higher than normally needed to quantitatively hydrolyze BCCP to BCCP(9100), only 30% of the protein was converted to a faster migrating biotin peptide that was shown in separate experiments to differ from BCCP(9100) by only a few amino acid residues. There was no apparent effect of subtilisin Carlsberg or subtilisin BPN' at lower concentrations (e.g., ratios of 1:10 or less). Similarly, BCCP(9100) showed the same stability to high levels of subtilisin. When BCCP(9100) and BCCP(9100) were incubated with trypsin, chymotrypsin or thermolysin (at ratios of 1:10 as described above) there was no apparent effect, further demonstrating their resistance to proteolysis.

In contrast to BCCP(9100) and BCCP(9100), BCCP(9100) was very susceptible to subtilisin degradation. Gel 3 (Fig. 8) shows a sample of 15 μg of untreated BCCP(9100). After treatment at 37° and pH 8 with a low level of subtilisin Carlsberg (5 ng, Gel 4) or a high level (500 ng, Gel 5), a progressive conversion of BCCP(9100) to a species of BCCP similar to BCCP(9100) and
BCCP(1900) was observed. Clearly, the forms of BCCP represented by BCCP(4200) and BCCP(2400) are very stable to further subtilisin degradation, while forms of BCCP such as BCCP(20400) are readily hydrolyzed to these smaller, more stable species. This suggests that the previously reported isolation of a mixture of small BCCP species (6, 9) can be explained by a subtilisin-type proteolysis of BCCP which did not go to completion.

**Characterization of BCCP(1900)**

**Isolation of BCCP(1900)**—Studies described above suggested that a small stable species of BCCP (referred to as BCCP(1900)), very similar in size to BCCP(4200), could be produced in high yields from crude or purified preparations of BCCP treated with subtilisin Carlsberg. In an effort to isolate milligram quantities of this protein we screened various BCCP preparations for suitability as starting material. Pilot studies were conducted on several partially purified preparations of [biotin-3H]BCCP (corresponding to the alumina Cy gel eluate, Step 3, as previously described (8)) to determine the degree of proteolysis with various levels of subtilisin Carlsberg. These preparations were treated with this protease at 37°C in 0.02 M Tris-Cl, pH 8.0; the reaction was terminated by the addition of 2 mM phenylmethylsulfonyl fluoride and the [3H]biotin labeled reaction products were analyzed by polyacrylamide gel electrophoresis as described under “Methods.” With these preparations of BCCP ratios of 1:300 to 1:1000 parts of protein to protease gave essentially quantitative hydrolysis to BCCP(1900) in 10 min. The details of a typical purification of [biotin-3H]BCCP(1900) from such a preparation of [biotin-3H]BCCP are described in the “Experimental Procedure.” After treatment of the preparation with subtilisin Carlsberg, the mixture was fractionated with ammonium sulfate, followed by Sephadex G-100 chromatography and preparative polyacrylamide gel electrophoresis (Table I). The purity of the final BCCP(1900) preparation was established by standard and sodium dodecyl sulfate polyacrylamide gel electrophoresis; in each case only a single protein band was detected, corresponding in migration position to authentic BCCP(1900).

**Isoelectric Focusing of BCCP(1900) and BCCP(4200)**—Samples of [biotin-3H]BCCP(1900) and [biotin-3H]BCCP(4200) were subjected to isoelectric focusing in 7.5% polyacrylamide gels in a pH gradient of 3 to 6 as described by Righetti and Drysdale (27). Duplicate gels were either sliced and analyzed for pH and radioactivity, or stained for protein. Examples of stained gels are shown in Fig. 9. Gel 1 contains 10 μg of BCCP(4200); Gel 2 contains 10 μg of BCCP(1900); Gel 3 contains 15 μg of a second preparation of BCCP(1900); and Gel 4 contains 8 μg of a separate preparation of BCCP(4200). All four protein samples show almost identical position in the pH gradient. Duplicate determinations of the isoelectric points for BCCP(1900) and BCCP(4200) gave average values of 4.46 and 4.49, respectively.

**Amino Acid Analysis of BCCP(1900)**—The data from amino acid analyses of BCCP(1900) are shown in Table III. Also shown for comparison is the composition of BCCP(4200) (6). The only difference between the two proteins is that BCCP(1900) contains 2 fewer alanine residues than BCCP(4200), for a total of 80 amino acid residues. These data confirm the similarity of these two proteins. It is not yet known whether the difference between these two proteins involves the amino or carboxyl terminal of the protein (or both).

**Physical and Kinetic Properties of BCCP(1900)**—Some of the physical and kinetic properties of BCCP(1900) were measured and compared to those of BCCP(4200). These data are summarized in Table IV. All shown for comparison are the molecular weights for BCCP(1900) and BCCP(4200) calculated from their amino acid compositions. A sedimentation coefficient of 1.02 S for BCCP(4200) was determined by sedimentation velocity measurements. This compares to a value of 1.04 S for BCCP(1900) (6). The biotin content of the protein was determined by two independent methods and the average value, 1 mol of biotin per 9600 g of protein correlates well with the residue molecular weight of 8942 calculated from the amino acid analysis, and the biotin content determined previously (6, 7) for BCCP(4200). BCCP(1900) contains 1 sulfhydryl residue as measured by amino acid analysis (Table III), and this residue cannot be titrated with 5,5′-dithiobis(2-nitrobenzoic acid) unless denaturants are added. Even in the presence of 1% SDS the reaction is much slower, taking approximately 20 min to react one sulfhydryl residue; in the presence of 1% SDS the reaction is much slower, taking approximately 20 min to reach completion. This property of the molecule is identical to that seen with BCCP(9100) (6, 8), and the titration curves (not shown) for BCCP(1900) and BCCP(9100) were superimposable.

---

**Table III**

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* Samples of BCCP(1900) were subjected to acid hydrolysis for the number of hours indicated as described under “Experimental Procedure.” The values shown represent the average of duplicate determinations.

* The total integral value was determined from the average of the 24-, 48-, and 72-hour hydrolysate values, except for serine and threonine, which were determined by extrapolation to zero time, half-cystine which was determined as indicated, and valine, for which the 72-hour hydrolysis value was used. In the case of isoleucine, a value approaching 7 residues per residue of biotin was obtained after a 96-hour hydrolysis; this slow release of isoleucine is due to the presence of at least 2 Leu-Leu sequences.

* Amino acid composition from Nervi et al. (6).

* Determined as cysteic acid on performic acid oxidized samples (24); average of two separate determinations.

* Determined spectrophotometrically by the method of Edelhoch (25).

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R. Radshaw, personal communication.
TABLE IV

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<td>0.89 (slow reaction)</td>
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a Some of these data taken from Nervi et al. (6).

b Value from amino acid analysis, including 1 residue of biotin (mol wt 244.3); there was no correction for loss of amide ammonia.

c Determined by Pronase digestion followed by an avidin binding assay as previously described (8).

The ability of BCCP(ae) to serve as a carboxyl acceptor in the biotin carboxylase reaction and as a carboxyl donor in the transcarboxylase reaction was tested in a reconstituted acetyl-CoA carboxylase reaction as previously described (6, 7). The K_M value for BCCP(ae) in both of these reactions was determined to be approximately 3 × 10^-3 M. This compares to values of 2 to 2.5 × 10^-5 M determined for BCCP(MN) (6, 7). The V_max values for both forms of BCCP in these reactions were essentially identical.

DISCUSSION

The previous studies of the BCCP component of E. coli acetyl-CoA carboxylase have been complicated by the isolation of multiple active forms of this protein (6-9) ranging in molecular weight from 45,000 to 90,100. Evidence was obtained to suggest that only the largest of these forms of BCCP is the native form (7, 8), and we postulated that all the smaller forms of BCCP were derived by proteolysis. Indeed, a comparison of the amino acid composition of the native BCCP (207 residues per polypeptide chain) with those of BCCP(MN) (82 residues) and BCCP(ae) (96 residues) was consistent with the idea that these smaller polypeptides were derived from the native chain. However, no direct evidence was obtained for the proteolytic event (or events) which presumably produced these smaller polypeptides, and we were initially unable to repeat the isolation of these small forms.

We are now able to provide evidence for a specific proteolytic modification of BCCP by subtilisin Carlsberg which produces a form of BCCP, BCCP(ae), differing from BCCP(ae) by only 2 amino acid residues. In other respects the two proteins are identical. In addition, a study of the kinetics of the subtilisin mediated production of BCCP(ae) from BCCP revealed that multiple intermediate forms of BCCP are produced at early stages of the proteolysis. With further proteolysis these intermediate forms are all converted to the smallest polypeptide, BCCP(ae), which is stable to further proteolysis and therefore accumulates. These intermediates are analogous to the multiple species of BCCP isolated earlier (6, 9) and provide an explanation for their existence. Presumably, during some stage of the previously reported isolation of the small BCCP species (6, 9), a limited proteolysis of the subtilisin type took place, degrading BCCP to the series of BCCP fragments illustrated diagrammatically in Fig. 1 (mix). The terminal proteolytic step was the conversion to the smallest of these polypeptides, BCCP(MN). Further support for this scheme was obtained by treating BCCP(MN), one of the isolated intermediates, with subtilisin and observing its quantitative conversion to a smaller BCCP which was similar, if not identical, to BCCP(ae) and BCCP(ae).

It was perhaps fortuitous that the isolated BCCP peptides were still capable of CO2 acceptor and donor activity in the acetyl-CoA carboxylase reactions, although with apparent K_M values 50- to 100-fold higher than those found with the native BCCP. However, there are several reported instances of the production or isolation of active proteolytic fragments of enzymes (e.g. References 37-41). One recent example is the DNA polymerase from E. coli which can be cleaved by subtilisin into two active fragments (31-34). In a classic example of the use of subtilisin to cleave a protein, Richards and Vithayathil (30) demonstrated that RNAase could be hydrolyzed into two inactive fragments, RNAse S and S peptide, which when recombined regained enzymatic activity. We hoped to isolate the fragment lacking biotin as well as BCCP(ae) after subtilisin treatment of BCCP, in order to test their interaction. However, we were unable to isolate any polypeptide lacking biotin which would correspond to this fragment. Apparently this fragment, if it is produced, is rapidly degraded by subtilisin. Perhaps, under different conditions, it can be isolated.

The weight of our evidence suggests that native BCCP contains a region which is sensitive to subtilisin. Cleavage at this point results in the production of a biotin containing polypeptide which is rapidly further degraded to a stable "core" polypeptide represented by BCCP(ae). A similar finding has been made in the case of the BCP component of the acetyl-CoA carboxylase from Pseudomonas citrinoviolacei.

We are still not sure exactly which protease caused the production of BCCP(ae) and other small forms in the previously reported isolation (6, 9). It could have been an endogenous protease from E. coli; several endo- and exoproteases have been recently identified in E. coli (29). The protease may also have been a contaminant. In either case it seems most likely that the protease involved was of the subtilisin type.

Note Added in Proof—After this manuscript was submitted we discovered that the terminal step in the purification of BCCP(ae), preparative acrylamide gel electrophoresis, can be conveniently avoided. Instead, the pure protein can be more easily obtained by crystallization of the concentrated Sephadex G-100 pool, using the procedure described for the crystallization of BCCP(9100) (Ref. 6).

Acknowledgments—The authors thank Alfred W. Alberts for his valuable advice, Drs. Ralph A. Bradshaw and Carole J. Coffee for help in performing amino acid analyses, and Miss Carmelita Lowry for performing the ultracentrifugation. We also thank Mrs. Kay Williams for excellent technical assistance.
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R. Ray Fall and P. Roy Vagelos


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