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Acetyl CoA Carboxylase: Isolation and Characterization of Native Biotin Carboxyl Carrier Protein

(high molecular weight/high activity)

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ABSTRACT **A large form of biotin carboxyl carrier protein ($BCCP_L$) has been isolated from extracts of *Escherichia coli*. It has a minimal molecular weight of 20,000, according to its behavior on sodium dodecylsulfate–polyacrylamide gel electrophoresis, and contains approximately 1 mol of biotin per 22,000 g of protein. $BCCP_L$ exhibits K_m values, in the biotin carboxylase and transcarboxylase half-reactions of acetyl CoA carboxylase, of 2×10^{-7} M and 4×10^{-7} M, respectively; these values are 50–100 times lower than those obtained with smaller forms of BCCP previously isolated. Electrophoresis of crude extracts of *E. coli* indicates that the major biotin-containing protein migrates at the same rate as $BCCP_L$, which suggests that $BCCP_L$ is the native form of BCCP in *E. coli*.**

Escherichia coli acetyl CoA carboxylase has been resolved into three functionally dissimilar subunits, including a biotin carboxyl carrier protein of low molecular weight (BCCP), a biotin carboxylase, and a transcarboxylase (1–3). The BCCP plays a central role in the carboxylation of acetyl CoA, as indicated in reactions *1–3:*

$$ATP + HCO_3^- + BCCP \underset{}{\overset{\text{biotin carboxylase, } Mn^{2+}}{\rightleftharpoons}} CO_2^-\text{-}BCCP + ADP + P_i \quad (1)$$

$$CO_2^-\text{-}BCCP + CH_3CO\text{-}SCoA \overset{\text{transcarboxylase}}{\rightleftharpoons} {}^-O_2CCH_2CO\text{-}SCoA + BCCP \quad (2)$$

$$\text{Sum: } ATP + HCO_3^- + CH_3CO\text{-}SCoA \rightleftharpoons {}^-O_2CCH_2COSCoA + ADP + P_i \quad (3)$$

BCCP is first carboxylated in a Mn^{2+}- and ATP-dependent reaction catalyzed by biotin carboxylase to form CO_2^--BCCP. The CO_2^--BCCP then donates its carboxyl group to acetyl CoA, forming malonyl CoA in a transcarboxylation reaction catalyzed by the transcarboxylase component. A species of *E. coli* BCCP has been purified to homogeneity and crystallized (4). This form of BCCP has a molecular weight of 9100 and contains approximately 1 mol of covalently-bound biotin per 10,000 g of protein; this small form of BCCP is referred to as $BCCP_S$. Another small BCCP, with a molecular weight of 10,300, was also isolated from *E. coli* extracts (4); it differs from $BCCP_S$ by an additional 14 aminoacid residues. Both small forms of BCCP are equivalent cofactors in the carboxylation and transcarboxylation reactions.

In addition to the small forms of BCCP, we found some evidence for the existence of a larger form of BCCP in extracts of *E. coli* prepared by a different procedure (4). This larger BCCP was significantly more active than the smaller forms of BCCP. Only small amounts of this material were isolated and the purified protein spontaneously dissociated into fragments of smaller molecular weight.

This communication describes the isolation of the larger, more active form of *E. coli* BCCP (which is referred to as $BCCP_L$), and presents data which indicate that it is the native form of *E. coli* BCCP.

MATERIALS AND METHODS

E. coli B $^3/_4$ log cells were obtained from Grain Processing Corp. *E. coli* strain PA 502 was obtained from Dr. F. Jacob. Acetyl CoA was synthesized by the method of Simon and Shemin (5). Yeast fatty acid synthetase was purified by the method of Lynen (6). $BCCP_S$ (MW 9100) was purified as previously described (4).

E. coli strain PA 502, a biotin auxotroph, was grown in the presence of [^{3}H]biotin (1.35 Ci/mmol; New England Nuclear) as previously described (2, 4). Small quantities of these cells were broken in a French pressure cell, and the disrupted cells were centrifuged at 48,000 $\times$ *g* for 30 min; the supernatant fraction was combined with crude extracts of *E. coli* B prepared as described earlier (1). Some of the 48,000 $\times$ *g* supernatant fraction was analyzed directly by disc gel electrophoresis.

Acetyl CoA carboxylase was resolved into E_a and transcarboxylase fractions as previously described (1). The transcarboxylase component (E_b) was further purified (3) to a specific activity of 4 units/mg protein; this preparation was free of biotin carboxylase and BCCP. Biotin carboxylase was purified free of BCCP and transcarboxylase by chromatography of an E_a preparation on DEAE-cellulose (4); the final preparation had a specific activity of 0.6 μmol of (+)-biotin carboxylated per min per mg protein.

$BCCP_L$ was purified from an E_a preparation by ammonium sulfate fractionation followed by Sephadex G-200 chromatography, adsorption and elution from calcium phosphate gel, ammonium sulfate precipitation with back extraction, and preparative disc gel electrophoresis. Full details of the purification procedure will be reported elsewhere. The purified $BCCP_L$ was free of biotin carboxylase and transcarboxylase activities.

Abbreviations: SDS, sodium dodecyl sulfate; $BCCP_S$ and $BCCP_L$, small and large forms of biotin carboxyl carrier protein.

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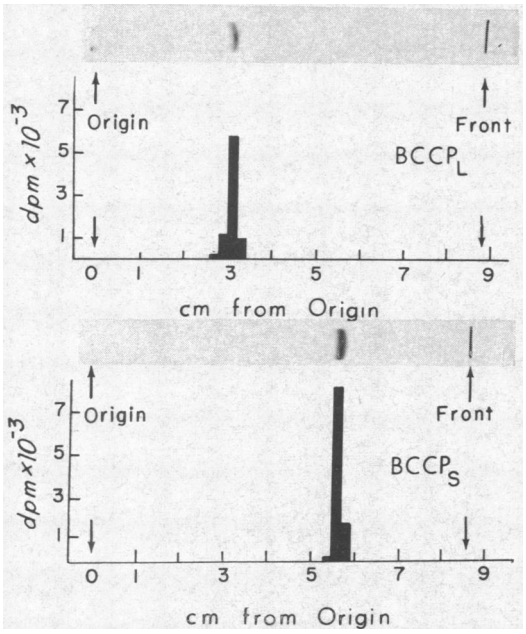

FIG. 1. Polyacrylamide gel electrophoresis of purified [^{3}H]-BCCP$_L$ and [^{3}H]BCCP$_S$. Approximately 8 μg of [^{3}H]BCCP$_L$ (10,400 dpm) and 10 μg of [^{3}H]BCCP$_S$ (16,500 dpm) were applied to 15% gels, which were afterwards sliced and counted.

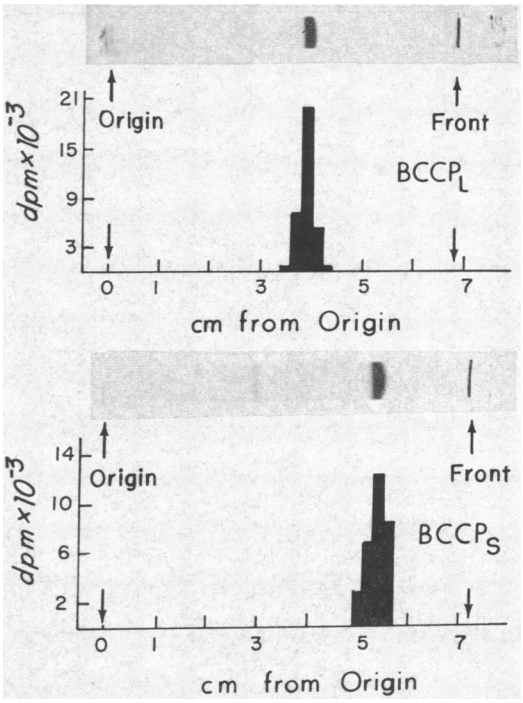

FIG. 2. SDS–polyacrylamide gel electrophoresis of purified [^{3}H]BCCP$_L$ (25 μg, 33,000 dpm) and [^{3}H]BCCP$_S$ (25 μg, 40,000 dpm). 15% gels containing 0.1% SDS (14).

Acetyl CoA carboxylase and biotin carboxylase activities were measured as previously described (4). Protein was determined by a microbiuret method (7) or by the method of Lowry *et al.* (8). The biotin content of BCCP was determined by stoichiometric carboxylation (1) with $H^{14}CO_3^-$ of the purified BCCP$_L$ and BCCP$_S$ in the presence of excess biotin carboxylase, and by the avidin-binding method of Green (9) as modified by Gerwin *et al.* (10).

Analytical disc gel electrophoresis was performed by the method of Davis (11). Gels of 15% acrylamide–0.15% bisacrylamide were used routinely to resolve the various forms of BCCP. Gels were fixed in 12.5% trichloroacetic acid and stained with Coomassie blue (12). Gels containing 0.1% sodium dodecyl sulfate (SDS) were run at pH 7.2 by the method of Weber and Osborn (13) and at pH 8.8 by the method of Laemmli (14); similar results were obtained with the two systems. SDS gels were fixed and stained as described previously (13). For the SDS electrophoresis of *E. coli* crude extract, samples were diluted in 2% SDS–2% 2-mercaptoethanol–0.02 M Tris·HCl (pH 8.0), heated at 37°C for 2 hr, and finally dialyzed overnight at 37°C against 0.1% SDS–0.1% 2-mercaptoethanol–0.01 M sodium phosphate, pH 7.0. Polyacrylamide gels containing [^{3}H]BCCP were sliced and dissolved in 30% H_2O_2 before liquid scintillation counting as previously described (1).

RESULTS

Isolation of BCCP$_L$

Attempts to purify larger amounts of BCCP$_L$ than were isolated earlier (4) were aided by the discovery that BCCP$_L$ is stabilized by storage in 20% glycerol, and consequently 20% glycerol was included in all buffers after the Sephadex G-200 step of the purification procedure (see *Methods*). [^{3}H]BCCP$_L$ was detected at each stage of the purification procedure by disc gel electrophoresis followed by radioactivity analysis. The results of such a procedure are illustrated in Fig. 1 for the purified BCCP$_L$. Shown for comparison is a similar gel electrophoresis of [^{3}H]BCCP$_S$. The two forms of BCCP are clearly resolved by this procedure. The purified BCCP$_L$ migrates as a single protein band and is free of any BCCP$_S$. Scanning of the gel shown in Fig. 1 indicated that the purified BCCP$_L$ is more than 95% pure; it is also free of any biotin carboxylase or transcarboxylase activity.

Characterization of BCCP$_L$

SDS disc gel electrophoresis of the two forms of BCCP revealed that the purified BCCP$_L$ migrates as a single band of higher molecular weight than BCCP$_S$ (MW 9,100) (Fig. 2). The extrapolated molecular weight for BCCP$_L$ from several such determinations such as those in Fig. 3 is approximately

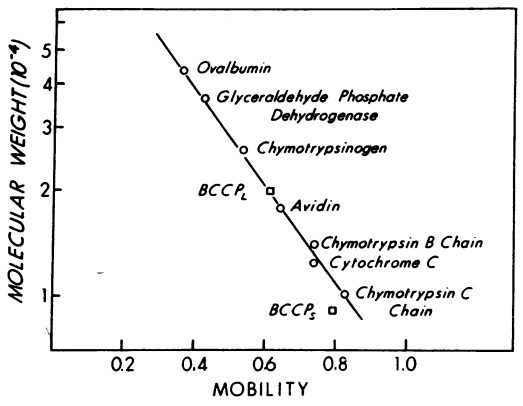

FIG. 3. Determination of the minimal molecular weight of BCCP$_L$ by SDS gel electrophoresis. 15% gels containing 0.1% SDS were used as described by Weber and Osborn (13). Protein samples of 10–20 μg were used. Molecular weight values for the standard proteins are from refs. 13 and 15.

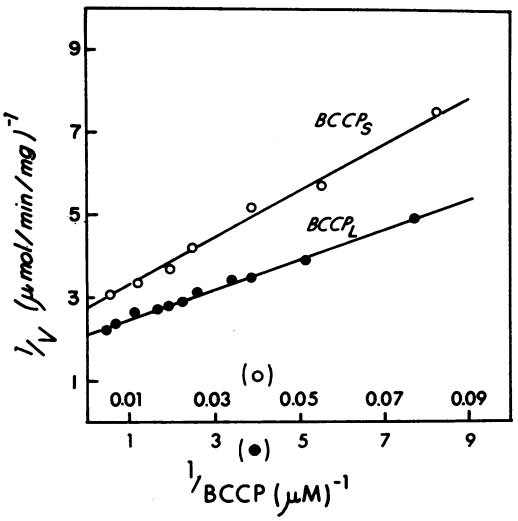

FIG. 4. Double reciprocal plot for $BCCP_L$ and $BCCP_S$ in the biotin carboxylase reaction. The spectrophotometric assay has been described previously (3). Each reaction mixture contained 0.004 unit of biotin carboxylase and 0.02 unit of transcarboxylase.

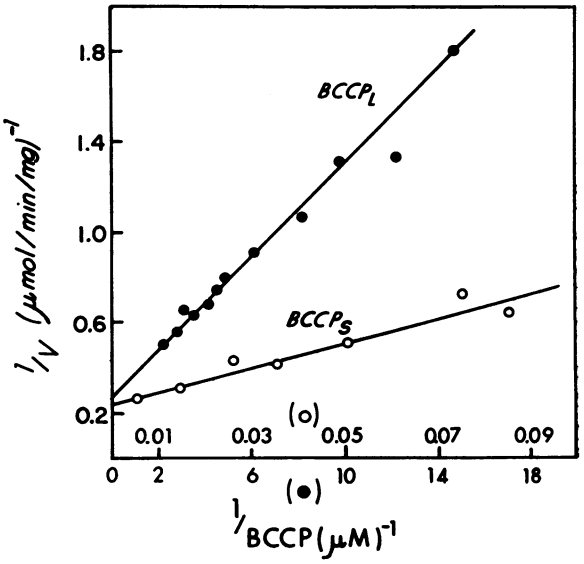

FIG. 5. Double reciprocal plot for $BCCP_L$ and $BCCP_S$ in the transcarboxylation reaction. The spectrophotometric assay was as in ref. 3 except that each reaction mixture contained 0.04 unit of biotin carboxylase and 0.002 unit of transcarboxylase.

20,000. $BCCP_S$ exhibits somewhat anomalous behavior on SDS gels and consistently migrates more slowly than expected for a polypeptide chain of molecular weight 9100 (see Fig. 3). $BCCP_L$ appeared to be incompletely dissociated when incubated in 2% SDS–2% 2-mercaptoethanol at 23°C for 1 hr, and two radioactive bands were detected corresponding to molecular weights of 26,000 and 20,000. However, heating at 37°C under these same conditions resulted in the appearance of a single radioactive band of molecular weight 20,000. These results indicate that under these conditions the minimal molecular weight of the $BCCP_L$ is approximately twice that of $BCCP_S$.

Attempts to determine the molecular weight of $BCCP_L$ in the absence of SDS on a calibrated Sephadex G-75 column gave variable results with molecular weight values ranging from 20,000 to 45,000. The cause of this anomalous behavior is not yet known. Unless glycerol was included in the elution buffer, the peak of recovered $BCCP_L$ exhibited several bands on subsequent disc gel electrophoresis.

The biotin contents of $BCCP_L$ and $BCCP_S$ were determined by stoichiometric carboxylation with $H^{14}CO_3^-$ in the presence of excess biotin carboxylase and by hydrolyzing both forms of BCCP with pronase and measuring released biotin by the avidin assay of Green (9) (Table 1). The $BCCP_L$ data, which were obtained with the purest preparation of $BCCP_L$ (>95% pure), indicate an average of 1 mol of biotin per 22,000 g of protein. The biotin content of $BCCP_S$ indicates approximately 1 mol of biotin per 9300 g of protein. These data independently confirm the minimal molecular weight value of 20,000 determined for $BCCP_L$ by SDS gel electrophoresis.

TABLE 1. *Biotin content of $BCCP_L$ and $BCCP_S$*

Preparation	Method	Biotin content (g protein/mol biotin)
$BCCP_L$	carboxylation	21,500
$BCCP_L$	pronase–avidin	22,200
$BCCP_S$	carboxylation	9,600
$BCCP_S$	pronase–avidin	9,000

Reconstitution of acetyl CoA carboxylase activity

The activity of the $BCCP_L$ in partial reactions *1* and *2* of acetyl CoA carboxylase was tested by spectrophotometric assay of malonyl CoA formation (2, 4) upon recombination of the three purified protein components: biotin carboxylase, transcarboxylase, and $BCCP_L$. Each protein was free of the other two (see *Methods*). The effect of BCCP concentration on biotin carboxylase activity (reaction *1*) was tested under conditions of limiting biotin carboxylase and excess transcarboxylase in the reaction mixtures. To test the effect of CO_2–BCCP concentration on the transcarboxylase reaction (reaction *2*), we studied reactions with an excess of biotin carboxylase and limiting transcarboxylase. The activities of $BCCP_L$ and $BCCP_S$ in reactions *1* and *2* are compared in the double reciprocal plots in Figs. 4 and 5. K_m values of 2×10^{-7} M and 2×10^{-5} M were found for $BCCP_L$ and $BCCP_S$, respectively, in the biotin carboxylase reaction (Fig. 4). In this reaction, a higher V_{max} value was obtained with $BCCP_L$. K_m values of 4×10^{-7} M and 2×10^{-5} M were found for $BCCP_L$ and $BCCP_S$, respectively, in the transcarboxylase reaction (Fig. 5). In this reaction, somewhat higher V_{max} values were obtained with $BCCP_S$ than $BCCP_L$. These data indicate that both the biotin carboxylase and transcarboxylase components of acetyl CoA carboxylase have a much higher affinity for $BCCP_L$ than for $BCCP_S$, which lends support to the notion that $BCCP_L$ is the native form of *E. coli* BCCP (see below).

Native form of *E. coli* BCCP

The discovery of $BCCP_L$ raises the question as to the form of BCCP present in *E. coli in vivo*. Disc gel electrophoresis of freshly prepared crude extracts of *E. coli* grown on [^{3}H]biotin, on a standard 15% gel at pH 8.9 (Fig. 6) and on an SDS gel (Fig. 7), shows that the great majority of protein-bound radioactive biotin in crude extracts migrates as $BCCP_L$ in both systems. A trace of faster-moving material is always

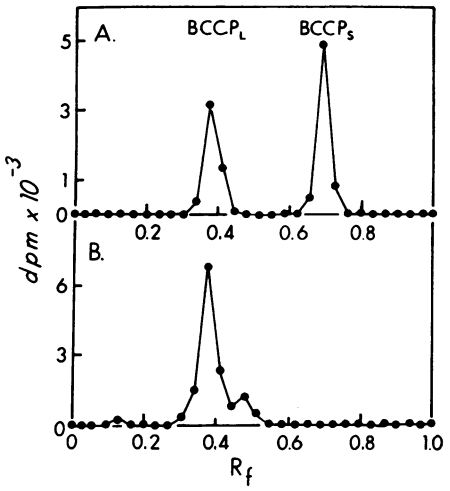

FIG. 6. Polyacrylamide gel electrophoresis of the [^{3}H]biotin-containing proteins in a crude *E. coli* extract. 15% gels were used as described in *Methods*. *A*, mixture of purified [^{3}H]BCCP$_L$ and [^{3}H]BCCP$_S$. *B*, freshly prepared *E. coli* extract; approximately 0.3 mg of protein (15,000 dpm).

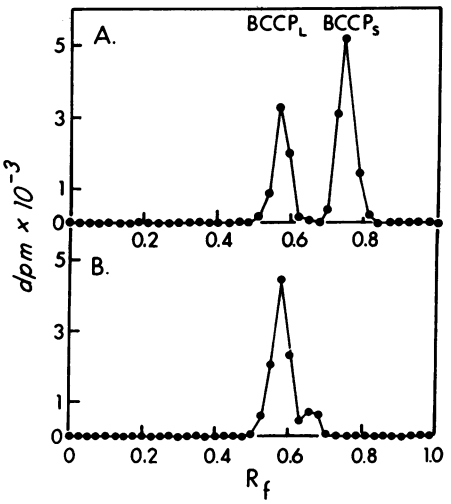

FIG. 7. SDS–polyacrylamide gel electrophoresis of the [^{3}H]biotin-containing proteins in a crude *E. coli* extract. 15% gels containing 0.1% SDS. *A*, mixture of purified [^{3}H]BCCP$_L$ and [^{3}H]BCCP$_S$. *B*, freshly prepared *E. coli* extract which was incubated in SDS (see *Methods*): approximately 0.3 mg of protein (15,000 dpm).

noted, but no BCCP$_S$ has been detected in freshly prepared crude extracts. Dialysis of crude extracts gives several faster-moving bands of ^{3}H-labeled protein on standard gels, which appear to be proteolysis products of BCCP$_L$. The conditions for the formation of BCCP$_S$ seem to be related to the repeated dialysis of the *E. coli* crude extract at high pH during the large-scale purification of BCCP$_S$ previously reported (4).

DISCUSSION

Examination of crude extracts of *E. coli* indicates that only one major biotin-containing protein exists, and this corresponds to BCCP$_L$. No evidence has been found for the natural occurrence of BCCP$_S$ or the slightly larger form of BCCP (MW 10,300), and we suspect that these smaller forms of BCCP result from the cleavage of BCCP$_L$ during the previously reported purification (4). The two smaller forms of BCCP probably result from hydrolysis of at least two different sensitive bonds in BCCP$_L$. Our studies have not ruled out the possibility that the smaller forms are naturally occurring, less active forms of BCCP that are in equilibrium with BCCP$_L$. However, this seems unlikely.

Perhaps the most significant feature of BCCP$_L$ is that it is active in both acetyl CoA carboxylase partial reactions at concentrations 50–100 times lower than those at which BCCP$_S$ is active. This is further support for the idea that the BCCP$_L$ represents the native form of BCCP. The low K_m values (2–4 $\times 10^{-7}$ M) are in the range expected for such a protein cofactor.

The earlier isolation of BCCP$_S$ is now readily understood. This protein was initially detected as a radioactive component of radioactive biotin-labeled fraction E$_a$. Since complete dissociation of the E$_a$ complex was desired, we selected dissociation and purification procedures which led to the isolation of the smallest radioactive peptide that was still active in the acetyl CoA carboxylase reaction. It is now apparent that these conditions also permitted proteolysis to occur.

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1. Alberts, A. W., and P. R. Vagelos, *Proc. Nat. Acad. Sci. USA*, **59**, 561 (1968).
2. Alberts, A. W., A. M. Nervi, and P. R. Vagelos, *Proc. Nat. Acad. Sci. USA*, **63**, 1319 (1969).
3. Alberts, A. W., S. G. Gordon, and P. R. Vagelos, *Proc. Nat. Acad. Sci. USA*, **68**, 1259 (1971).
4. Nervi, A. M., A. W. Alberts, and P. R. Vagelos, *Arch. Biochem. Biophys.*, **143**, 40 (1971).
5. Simon, E. J., and D. Shemin, *J. Amer. Chem. Soc.*, **75**, 3520 (1953).
6. Lynen, F., in *Methods in Enzymology*, ed. J. M. Lowenstein (Academic Press, New York, 1969), Vol. XIV, p. 17.
7. Munkres, K. D., and F. M. Richards, *Arch. Biochem. Biophys.*, **109**, 466 (1965).
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9. Green, N. M., in *Methods in Enzymology*, ed. D. B. McCormick and L. D. Wright (Academic Press, New York, 1970), Vol. XVIII, p. 418.
10. Gerwin, B. I., B. E. Jacobson, and H. G. Wood, *Proc. Nat. Acad. Sci. USA*, **64**, 1315 (1969).
11. Davis, B. J., *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
12. Chrambach, A., R. A. Reisfeld, M. Wyckoff, and J. Zaccari, *Anal. Biochem.*, **20**, 150 (1967).
13. Weber, K., and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
14. Laemmli, U. K., *Nature*, **227**, 680 (1970).
15. Dunker, A. K., and R. R. Rueckert, *J. Biol. Chem.*, **244**, 5074 (1969).