*ACETYL CoA CARBOXYLASE, II. DEMONSTRATION OF BIOTIN-PROTEIN AND BIOTIN CARBOXYLASE SUBUNITS**

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Abstract.—Previous work has shown that *Escherichia coli* acetyl CoA carboxylase is composed of two dissimilar protein components, E_a which contains covalently bound biotin and forms E_a-CO_2^- from HCO_3^- and ATP, and E_b which is involved in the transfer of the carboxyl group from E_a-CO_2^- to acetyl CoA, forming malonyl CoA. E_a has been dissociated into two subunits at pH 9. One subunit, designated biotin carboxylase, catalyzes a model reaction, the ATP-dependent carboxylation of free (+)-biotin. The other subunit contains covalently bound biotin which is carboxylated by the biotin carboxylase in the course of acetyl CoA carboxylation.

Acetyl CoA carboxylase of *Escherichia coli* has been shown to be separable into two protein fractions, E_a and E_b, which are involved in the carboxylation reaction as follows:[1-3]

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

$$CO_2^-\text{-biotin-}E_a + CH_3CO\text{-SCoA} \overset{E_b}{\rightleftharpoons} {}^-O_2CCH_2CO\text{-SCoA} + \text{biotin-}E_a \quad (2)$$

$$\text{Sum: ATP} + HCO_3^- + CH_3CO\text{-SCoA} \overset{\text{biotin-}E_a,\ E_b,\ Mn^{2+}}{\rightleftharpoons} {}^-O_2CCH_2CO\text{-SCoA} + \text{ADP} + P_i \quad (3)$$

Thus, it was shown that E_a contains covalently bound biotin which is carboxylated in the presence of ATP and Mn^{2+} forming carboxybiotin-E_a (reaction 1). The second protein fraction, E_b, is involved specifically in the transfer of the carboxyl group from the biotin protein to the acceptor, acetyl CoA, forming malonyl CoA (reaction 2).

Two biotin proteins, β-methylcrotonyl CoA carboxylase[4-6] purified from *Mycobacterium* and *Achromobacter* and acetyl CoA carboxylase of avian liver[7] catalyze the carboxylation of free (+)-biotin according to reaction 4:

$$\text{ATP} + HCO_3^- + (+)\text{-biotin} \overset{Mn^{2+}}{\rightleftharpoons} CO_2^-\text{-}(+)\text{-biotin} + \text{ADP} + P_i \quad (4)$$

Thus, it was no surprise to find that E_a, a component of *E. coli* acetyl CoA carboxylase, also catalyzes free (+)-biotin carboxylation, and this enzymatic activity has been utilized in further fractionation of E_a. E_a has now been dissociated into two components: a low-molecular-weight protein which contains covalently bound biotin and a larger protein which contains no biotin but which catalyzes the carboxylation of free (+)-biotin.

Materials.—*E. coli* B 1/4 log cells were obtained from Grain Processing Corp. *E. coli* strain PA 502 was obtained from Dr. F. Jacob. Sodium ^{14}C-bicarbonate was obtained

from New England Nuclear Corp. and ^{14}C-(+)-biotin from Nuclear-Chicago Corp. ATP and CoA were purchased from P-L Biochemicals, Inc. and silica gel Chromagram from Eastman. Avidin was obtained from Nutritional Biochemicals Corp. and alumina Cγ, calcium phosphate gel, lactic dehydrogenase, pyruvate kinase, and phosphoenolpyruvate from Sigma Chemical Co. Acetyl CoA was synthesized by the method of Simon and Shemin.[8] 1'-*N*-methoxycarbonyl biotin methyl ester was a generous gift from Dr. M. D. Lane.

Methods.—E. coli strain PA 502, a biotin auxotroph, was grown in a New Brunswick F250 Fermacel fermentor. The medium, 200 l of 63[9] at pH 7.0, contained, in addition to salts, 800 ml glycerol, 40 gm each of proline, histidine, and arginine, as well as 20.0 mg thiamine and 2.0 mg of ^{14}C-biotin (6.3 μc/μmole). After 24 hr of growth with aeration at 37°, the cells were harvested and stored as a paste at −20°.

E_a and E_b were prepared as previously described.[2] E_a was further purified by adsorption onto calcium phosphate gel and subsequent elution with 0.2 *M* potassium phosphate buffer, pH 7.8.

The *radioactive assay of acetyl CoA carboxylase* was as described previously.[2] In some cases a coupled spectrophotometric assay was used for E_a. The reaction mixture contained 55 mM imidazole-HCl buffer, pH 6.7, 0.44 mM $MnCl_2$, 0.44 mM adenosine 5'-triphosphate (ATP), 0.3 mM acetyl CoA, 30 mM $KHCO_3$, 0.33 mM phosphoenolpyruvate, 0.22 mM DPNH, 10 μg lactic dehydrogenase, 10 μg pyruvate kinase, and 0.01 unit E_b in a volume of 90 μl. The decrease in optical density at 340 mμ due to the oxidation of DPNH dependent on the addition of E_a (0–0.001 unit) was recorded on a Gilford model 2400 spectrophotometer at 30°. *Biotin carboxylase* was assayed spectrophotometrically in an identical manner to that of acetyl CoA carboxylase except that acetyl CoA and E_b were omitted and 90.0 mM (+)-biotin was included in the reaction mixture. *Disc gel electrophoresis* was performed according to the method of Davis[10] at pH 9.0 with a Canalco model 66 apparatus. Gels were sliced with a Canalco lateral gel slicer. *Sucrose gradient centrifugation* between 5 and 20% was by the method of Martin and Ames.[11] A Spinco L2 65B ultracentrifuge with an SW56 rotor was used at 56,000 rpm. Fractions were collected from the top of each tube with an ISCO model 180 density gradient fractionator. *Protein* was determined by a microbiuret method.[12] *Radioactivity* was counted in 10 ml of Bray's solution[13] in a Packard Tri-Carb liquid scintillation counter.

Results.–The carboxylation of free (+)-biotin: The report of Stoll *et al.*[7] that pigeon liver acetyl CoA carboxylase catalyzes the carboxylation of free (+)-biotin prompted the testing of the individual fractions of *E. coli* acetyl CoA carboxylase for this activity. As seen in Table 1 (Experiment 1), a highly purified preparation of the E_a fraction of *E. coli* acetyl CoA carboxylase effectively catalyzes the carboxylation of free (+)-biotin. In the coupled assay system used there was an absolute requirement for (+)-biotin and a partial requirement for $KHCO_3$ for the oxidation of DPNH in the presence of ATP, $MnCl_2$, lactic dehydrogenase, phosphoenolpyruvate, and pyruvate kinase. E_b neither affected the rate of the reaction in the presence of E_a nor did it catalyze the reaction in the absence of E_a. When acetyl CoA was substituted for (+)-biotin (Expt. 2), E_b was also required for the oxidation of DPNH. As seen, the rate of acetyl CoA carboxylation was approximately 50 per cent the rate of free (+)-biotin carboxylation. The ratio of these two activities remained fairly constant through several steps of purification, although, as will be described below, acetyl CoA carboxylase activity was lost under some conditions in which biotin carboxylase activity was relatively unaffected.

Free biotin carboxylation by fraction E_a was linear for several hours at 30° with saturating levels of (+)-biotin and bicarbonate. It appeared to stop only

when DPNH was exhausted; addition of further amounts of DPNH allowed the reaction to proceed. As with the two previous enzymes[4-7] which catalyze this reaction a high concentration of (+)-biotin is required. The K_m for (+)-biotin is approximately 0.02 M. Levels of (+)-biotin up to 0.13 M, the highest tested, showed no inhibitory effect. E_b did not appear to have any effect on the K_m for biotin.

Effect of avidin on the biotin carboxylase reaction: It has been previously demonstrated[1-3] that the E_a fraction of *E. coli* acetyl CoA carboxylase contains covalently bound biotin. In order to determine if this bound biotin is involved in the carboxylation of free (+)-biotin, avidin, the specific biotin binding protein from egg white was employed. As shown in Table 2 acetyl CoA carboxylation was completely inhibited when E_a was incubated with avidin. This inhibition was prevented when (+)-biotin was mixed with the avidin before the enzyme was added. Under these same conditions there was no inhibition of the biotin

TABLE 1. *Requirements for carboxylation.*

Expt. 1	DPNH oxidized (mμmoles/min)
Complete system	0.91
$-E_a$	0
−biotin	0
$-KHCO_3$	0.11
$+E_b$	0.90
Expt. 2	
Complete system	0.48
$-E_a$	0
$-E_b$	0.02
−acetyl CoA	0

The spectrophotometric assays were carried out as described in *Methods*. Expt. 1 refers to the assay for biotin carboxylase and Expt. 2 to the assay for acetyl CoA carboxylase. In each experiment, 4 μg of E_a (alumina Cγ eluate) and 45 μg of E_b were added.

TABLE 2. *Effect of avidin on acetyl CoA carboxylase and biotin carboxylase activity.*

Avidin units	Acetyl CoA carboxylase (%)	Biotin carboxylase (%)
0	100	100
0.08	61.2	...
0.24	26.0	100
0.40	13.4	...
0.80	3.1	100
0.80*	98.5	100

E_a, (0.01 unit of acetyl CoA carboxylase activity) was incubated at 25° in a vol of 25 μliters with the units of avidin indicated. After 5 min, 0.2 μmole of biotin was added to remove excess avidin. Aliquots of each were assayed in the radioactive assay for acetyl CoA carboxylase in the presence of added E_b and in the spectrophotometric assay for biotin carboxylase as described in *Methods*.

* Biotin added before the addition of enzyme.

carboxylase reaction by any level of avidin tested. The amount of avidin used in these experiments was shown to inhibit the stoichiometric carboxylation of enzyme-bound biotin (reaction 1) as well as the transfer of the carboxyl group of carboxybiotin protein to acetyl CoA.[1-3] Thus, these and other experiments to be described below preclude the possibility that protein-bound biotin is involved in the carboxylation of free (+)-biotin.

Identification of the product of the reaction: The product of the reaction between free (+)-biotin and $H^{14}CO_3^-$ was identified as 1′-*N*-^{14}C-carboxybiotin. After gassing with CO_2 to remove unreacted $H^{14}CO^{3-}$, methylation was carried out with diazomethane as described by Lynen *et al.*[5] to convert the remaining free biotin and the reaction product to a mixture of biotin methyl ester and 1′-*N*-methoxycarbonyl biotin methyl ester, respectively. Thin-layer chromatography was carried out on Eastman silica gel Chromagram sheets with benzene-ethanol (90:10) as solvent. Authentic 1′-*N*-methoxycarbonyl biotin methyl

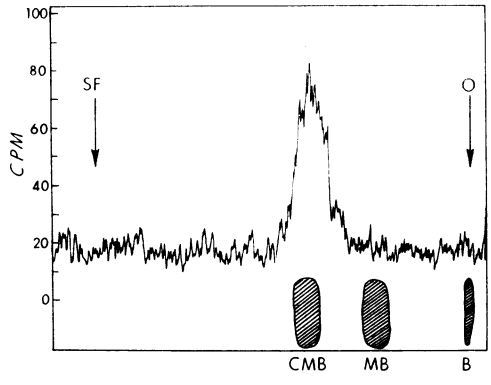

FIG. 1—Identification of the product of the biotin carboxylase reaction. B, (+)-biotin; MB, biotin methyl ester; CMB, methoxycarbonyl biotin methyl ester; O, origin; SF, solvent front.

ester and free biotin were included as standards in the chromatography. Figure 1 presents a radioisotope scan of such a chromatogram. Superimposed at the bottom of the scan is a tracing of the location of the spots obtained after spraying the thin-layer plate with potassium permanganate. As can be seen, the two reference compounds and the methyl ester of free (+)-biotin derived from the incubation mixture were well separated from each other. Under the conditions employed, free biotin remained at the origin while biotin methyl ester had an Rf of 0.24 and 1′-*N*-methoxycarbonyl biotin methyl ester an Rf of 0.43. Only one radioactive region was seen and it was associated with 1′-*N*-methoxycarbonyl biotin methyl ester. Thus the carboxylation of free (+)-biotin by fraction E_a of *E coli* acetyl CoA carboxylase yields 1′-*N*-carboxybiotin.

Dissociation of fraction E_a: Studies of E_a were greatly hampered by extreme lability of this fraction during procedures of protein fractionation. This problem was solved by the discovery that E_a is quite stable when maintained in 20 per cent glycerol. To facilitate further characterization of E_a, this fraction was purified from extracts of *E. coli* strain PA 502, a biotin auxotroph which was grown in the presence of ^{14}C-biotin. The covalently bound biotin could be followed even under conditions where catalytic activity was lost. Disc gel electrophoresis of highly purified E_a labeled with (+)-biotin revealed two major protein bands which were present at all stages of purification. Two aliquots of E_a, purified through the calcium phosphate disc gel, were electrophoresed at pH 9. One gel was stained for protein, scanned at 650 mμ, and then sectioned. The sections were dissolved in 30 per cent H_2O_2 and counted. The other gel was sectioned without staining and each section was eluted with 0.05 *M* potassium phosphate buffer, pH 7.3. The eluates were assayed for biotin carboxylase, acetyl CoA carboxylase in the presence of added E_b, and radioactivity. The results are shown in Figure 2. Although biotin carboxylase was associated with the slower moving band with the peak about 1 cm from the top of the gel, no E_a activity was detected in the acetyl CoA carboxylase assay. Radioactivity determined on either the stained or the unstained sections was identical and was present only in the fastest moving protein band. Thus, disc gel electrophoresis of E_a leads to the dissociation E_a into two protein components; one catalyzes the carboxylation of free (+)-biotin, and the other contains protein-bound biotin. Acetyl CoA carboxylase activity of E_a was not recovered from this procedure even when fractions were recombined.

Sucrose density gradient centrifugations: Preliminary investigations of ^{14}C-bio-

tin-labeled E_a using sucrose gradient centrifugation or Sephadex G-200 filtration indicated that enzymatic activity was entirely lost during these procedures and that all the radioactivity of the preparation was associated with a low-molecular-weight protein. When ^{14}C-biotin E_a was subjected to sucrose density centrifugation at pH 7.8 in the presence of 20 per cent glycerol (Fig. 3), both acetyl CoA carboxylase (in the presence of added E_b) and biotin carboxylase activities were recovered and these were present together in fractions 10 to 18 which also contained ^{14}C-biotin radioactivity. However, it is apparent that some radioactivity was also present in a slowly sedimenting peak of radioactivity in fractions 1 to 5. Thus, although some intact active E_a was recovered under these conditions (fractions 10 to 18), a portion of the biotin protein had dissociated from the more rapidly sedimenting active protein.

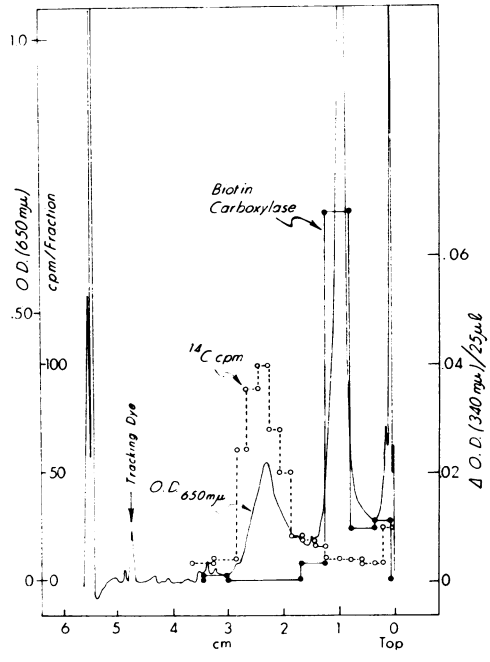

FIG. 2—Disc gel electrophoresis of E_a. Two 20-μl aliquots of ^{14}C-biotin-labeled E_a (0.1 mg, 1000 cpm, 0.08 unit acetyl CoA carboxylase, and 0.15 unit biotin carboxylase per aliquot) in 20% glycerol were layered over a 0.5 cm stacking gel and a 5.5-cm 7% separating gel and electrophoresed for 1 hr at 4 ma per tube. The gels then were treated as described in the text.

In order to separate the biotin protein from the biotin, carboxylase as had been accomplished with gel electrophoresis at pH 9, sucrose density gradient centrifugations were carried out at this pH. Thus, 1.2 ml of E_a containing 5×10^5 cpm of ^{14}C-biotin, 32 units of acetyl CoA carboxylase (assayed in the presence of added E_b) and 77 units of biotin carboxylase were centrifuged at pH 9.0 in a 5–20 per cent sucrose gradient containing 20 per cent glycerol in six buckets for 24 hours. The corresponding fractions from each bucket were pooled, and aliquots of the pooled fractions were counted and assayed for both carboxylase activities. Radioactivity was present as a broad peak in the upper fractions but it was smeared throughout most of the fractions. Although biotin carboxylase was found in a single sharp peak in good yield, only a small fraction of the acetyl CoA carboxylase activity, measured in the presence of added E_b, was recovered. Since the diffusely spread radioactivity extended into those fractions containing biotin carboxylase, the latter fractions were pooled, concentrated, and centrifuged again at pH 9, as above. No acetyl CoA carboxylase activity was recovered from this centrifugation. The biotin carboxylase had further dissociated from the biotin protein since the peak of enzymatic activity contained less radioactivity. Therefore, this procedure was repeated again in order to

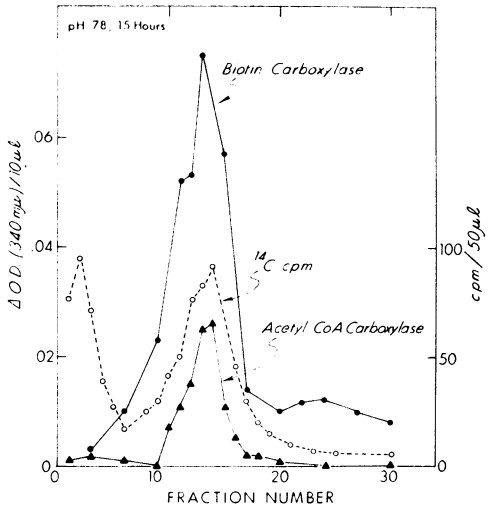

FIG. 3—Sucrose density gradient centrifugation of E_a at pH 7.8. E_a (0.1 mg, 1000 cpm, 0.08 unit acetyl CoA carboxylase, 0.15 unit biotin carboxylase) in 100 μl 0.02 *M* potassium phosphate buffer and 20% glycerol was layered over 4 ml of a 5–20% sucrose gradient containing 0.02 *M* potassium phosphate buffer pH 7.8, and 20% glycerol and centrifuged for 15 hr at 5°. Fractions were collected from the top. Aliquots were counted (O– –O), assayed for acetyl CoA carboxylase in the presence of E_b (▲—▲), and biotin carboxylase (●—●) as described in *Methods*.

further resolve the two proteins. Figure 4 indicates that this final centrifugation yielded biotin carboxylase (approximate *S* value of 5.4) containing very little of the biotin protein which now sedimented as a sharp peak with an approximate *S* value of 1.3.

A summary of the ratios of E_a activity in the acetyl CoA carboxylase assay to biotin carboxylase and of biotin carboxylase to ^{14}C-biotin-protein radioactivity for the various preparations from the alumina Cγ step through the third sucrose gradient is shown in Table 3. It can be seen that the ratios did not change significantly between the alumina Cγ and the calcium phosphate gel steps where the two specific enzymatic activities increased approximately eightfold. However, E_a activity in the acetyl CoA carboxylase assay was largely lost during the

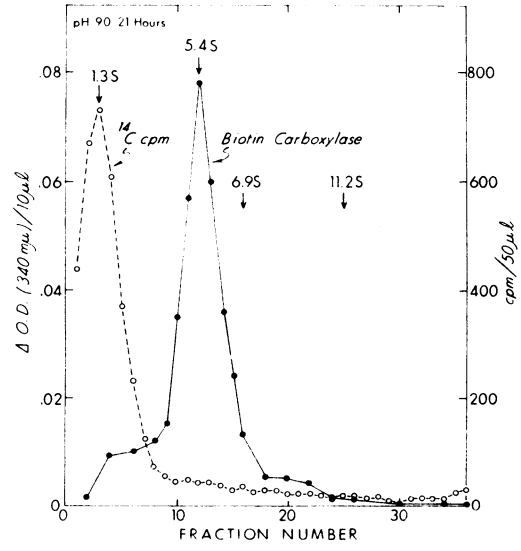

FIG. 4—Sucrose density gradient centrifugation of biotin carboxylase at pH 9.0. Biotin carboxylase obtained from two successive centrifugations of E_a at pH 9.0 as described in the text was centrifuged for 21 hr at 10° in a 5–20% linear sucrose gradient containing 0.02 *M* Tris-HCl buffer, pH 9.0, and 20% glycerol. Fractions were collected from the top. Aliquots were counted (O– –O) and assayed for biotin carboxylase (●—●) as described in *Methods*. Also included in this centrifugation were lactic dehydrogenase (6.9*S*) and catalase (11.2*S*). The *S* values indicated for the radioactive peak and biotin carboxylase peak were calculated from the positions of the marker proteins.

TABLE 3. *Resolution of biotin carboxylase from biotin protein.*

Preparation	Acetyl CoA carboxylase* / Biotin carboxylase†	Biotin carboxylase $\left(\frac{\Delta OD}{min}\right)$† / ^{14}C-biotin protein (cpm)§
Al Cγ eluate	0.68	0.0007
Calcium phosphate gel eluate	0.48	0.0010
1st sucrose gradient	0.04	0.0038
2nd sucrose gradient	0	0.0068
3rd sucrose gradient	0	0.0133

* E_a activity in the acetyl CoA carboxylase coupled spectrophotometric assay with added E_b.
† Biotin carboxylase assayed as described in *Methods*.
§ Radioactivity of ^{14}C-biotin protein.

first sucrose centrifugation, whereas the ratio of biotin carboxylase to biotin-protein radioactivity increased about 20-fold between the alumina Cγ eluate and the preparation recovered from the third centrifugation. The over-all recovery of biotin carboxylase was approximately 25 per cent. Thus, the ratio of this activity to biotin-protein radioactivity could have been higher if the enzymatic activity were more stable. Instability of the biotin carboxylase was indicated by the finding that the enzyme activity decreased during storage after isolation from the sucrose gradients unless the dilute protein solutions were concentrated by ultrafiltration.

Discussion.—*E. coli* acetyl CoA carboxylase is composed of three proteins. One of these, designated biotin carboxylase, catalyzes a model reaction, the ATP-dependent carboxylation of (+)-biotin. It is apparent that this protein contains the sites for ATP, Mn^{2+}, bicarbonate, and the noncovalent site of (+)-biotin since it catalyzes the synthesis of carboxybiotin in the absence of the biotin subunit. The availability of this enzymatically active subunit of acetyl CoA carboxylase should facilitate studies of the mechanism of carboxybiotin formation and its function in the over-all reaction.

The biotin subunit, which dissociates from E_a at pH 9, contains covalently bound biotin and it has no known catalytic activity. It is this covalently bound biotin which is carboxylated by the biotin carboxylase subunit in the course of acetyl CoA carboxylation. The biotin protein in many ways resembles ACP,[14] the acyl carrier protein. Both are small proteins with S values of approximately 1.3. Both are components of complex enzyme systems which are readily dissociable in *E. coli* but which are firmly associated in other organisms. Both have covalently bound prosthetic groups, biotin in one case and 4′-phosphopantetheine in the other. The prosthetic groups of both proteins function in their respective reactions as covalent substrate binding sites. Thus, the biotin protein can be considered a carboxyl carrier protein.

The third protein of acetyl CoA carboxylase, E_b, is specifically involved in the transfer of the carboxyl group of carboxybiotin protein to the acceptor, acetyl CoA.[1-3] Although it is reasonable to assume that E_b contains the acetyl CoA binding site, the precise role of this subunit has not yet been elucidated.

It is apparent from recent reports of Wood and his coworkers[15-17] that the structure of *E. coli* acetyl CoA carboxylase is not unique as a biotin protein. They have found that the methylmalonyl-CoA:oxaloacetate transcarboxylase can be dissociated into dissimilar subunits at pH 8. One fraction contains cobalt and appears to be the pyruvate binding site, while the other fraction contains covalently bound biotin. Activity is recovered when the two fractions are combined at pH 5. The biotin-containing subunit can in turn be dissociated further into two smaller subunits. One of these contains biotin and has an S value of 1.3. Thus, although biotin carboxylase activity has not been demonstrated in this preparation, the structure of transcarboxylase appears to be quite analogous to *E. coli* acetyl CoA carboxylase.

In the light of these findings, it is tempting to postulate that all biotin enzymes might be composed of three different subunits: a biotin protein, a biotin carboxylase, and a subunit specifying the acceptor molecule. It is also possible that various biotin enzymes will contain similar E_a subunits and will differ from each other only in containing different E_b subunits which confer acceptor specificity on the enzyme complex.

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