The Biotin Domain Peptide from the Biotin Carboxyl Carrier Protein of *Escherichia coli*  
Acetyl-CoA Carboxylase Causes a Marked Increase in the Catalytic Efficiency of Biotin Carboxylase and Carboxyltransferase Relative to Free Biotin*

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Acetyl-CoA carboxylase catalyzes the first committed step in the biosynthesis of long-chain fatty acids. The *Escherichia coli* form of the enzyme consists of a biotin carboxylase activity, a biotin carboxyl carrier protein, and a carboxyltransferase activity. The C-terminal 87 amino acids of the biotin carboxyl carrier protein (BCCP87) form a domain that can be independently expressed, biotinylated, and purified (Chapman-Smith, A., Turner, D. L., Cronan, J. E., Morris, T. W., and Wallace, J. C. (1994) *Biochem. J.* 302, 881–887). The ability of the biotinylated form of this 87-residue protein (holo-BCCP87) to act as a substrate for biotin carboxylase and carboxyltransferase was assessed and compared with the results with free biotin. In the case of biotin carboxylase holoBCCP87 was an excellent substrate with a *Km* of 0.16 ± 0.05 mM and *Vmax* of 1000.8 ± 182.0 min⁻¹. The *VK* or catalytic efficiency of biotin carboxylase with holoBCCP87 as substrate was 8000-fold greater than with biotin as substrate. Stimulation of the ATP synthesis reaction of biotin carboxylase where carbamyl phosphate reacted with ADP by holoBCCP87 was 5-fold greater than with an equivalent amount of biotin. The interaction of holoBCCP87 with carboxyltransferase was characterized in the reverse direction where malonyl-CoA reacted with holoBCCP87 to form acetyl-CoA and carboxyholoBCCP87. The *Km* for holoBCCP87 was 0.45 ± 0.07 ms while the *Vmax* was 2031.8 ± 231.0 min⁻¹. The *VK* or catalytic efficiency of carboxyltransferase with holoBCCP87 as substrate is 2000-fold greater than with biotin as substrate.

The first committed step in the biosynthesis of long-chain fatty acids in all animals, plants, and bacteria is catalyzed by acetyl-CoA carboxylase (1). The reaction catalyzed by acetyl-CoA carboxylase involves two separate reactions shown in Scheme 1.

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\begin{align*}
\text{HCO}_3^- + \text{Mg-ATP} + \text{Enzyme-Biotin} & \rightarrow \text{Enzyme-Biotin-CO}_2^- + \text{Mg-ADP} + \text{Pi} \\
\text{Acetyl-CoA} & \rightarrow \text{Enzyme-Biotin} + \text{Malonyl-CoA}
\end{align*}
\]

**Scheme 1**

Acetyl-CoA carboxylase is composed of three different components which allows it to carry out these two distinct reactions. The biotin carboxylase component catalyzes the first half-reaction which involves the phosphorylation of bicarbonate by ATP to form a carboxyphosphate intermediate, followed by transfer of the carboxyl group to biotin to form carboxybiotin. *In vivo*, biotin is attached to the biotin carboxyl carrier protein (designated as Enzyme-Biotin in Scheme 1) via an amide bond between the valeric acid side chain of biotin and the ε-amino group of a specific lysine residue. In the second reaction, catalyzed by carboxyltransferase, the carboxyl group is transferred from biotin to acetyl-CoA to form malonyl-CoA. Animals contain all three of these components on one polypeptide chain (2). In contrast, these three different proteins are on separate polypeptides in the *Escherichia coli* form of acetyl-CoA carboxylase (3). Each protein of bacterial acetyl-CoA carboxylase can be isolated, and the biotin carboxylase and carboxyltransferase have been shown to retain activity (4). Moreover, both biotin carboxylase and carboxyltransferase are able to utilize free biotin as a substrate instead of biotin linked to the biotin carboxyl carrier protein, thereby simplifying kinetic analysis of these two enzymes (4). As such, the biotin carboxylase and carboxyltransferase components of *E. coli* acetyl-CoA carboxylase have long served as model systems for mechanistic studies of biotin-dependent carboxylases. *In vivo*, however, the biotin carboxyl carrier protein (BCCP),† rather than free biotin, is the natural substrate for biotin carboxylase and carboxyltransferase.

Initial purification procedures of the BCCP revealed two forms. One form was intact BCCP (156 residues), and the other was a 9.1-kDa fragment of BCCP which corresponded to the last 82 residues of BCCP and contained the biotin moiety (5). It was subsequently shown that treatment of BCCP with subtilisin Carlsberg produced the 9.1-kDa fragment which was stable, suggesting it was a domain of intact BCCP (6). With the advent

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Characterization of BCCP87

The three-dimensional structure of the 9.1-kDa fragment of BCCP has been determined by x-ray crystallography which revealed that the lysine residue to which biotin is attached is located in a turn connecting two δ-strands (9). The biotin moiety hydrogen bonds to a “thumb-like” protrusion of the protein that causes the biotin molecule to be conformationally restricted. The three-dimensional structure of apoBCCP87 determined by NMR spectroscopy shows small conformational differences in the turn containing the biotinylated lysine, with this region having a significantly higher degree of flexibility in the apo protein (10–12). Chemical modification and proteolysis studies revealed a subtle overall conformational difference between the apo and holo forms of BCCP87 (13), and this has been confirmed recently by NMR experiments which indicate that differences in side chain packing produce a less stable protein prior to biotinylation (10).

Despite the considerable amount of work done on the biotin domain of BCCP, it is surprising that it has never been characterized as a substrate for the biotin carboxylase and carboxyltransferase components of E. coli acetyl-CoA carboxylase. In this note we assess the ability of holoBCCP87 to act as a substrate for biotin carboxylase and carboxyltransferase. We show that, compared with free biotin, holoBCCP87 increases the catalytic efficiency of biotin carboxylase and carboxyltransferase 8000- to 2000-fold, respectively.

MATERIALS AND METHODS

Chemicals and Enzymes—Biotin carboxylase was isolated from a strain of E. coli that overexpresses the gene coding for the enzyme (14). Purification was accomplished using a histidine-tag attached to the N terminus of the enzyme and nickel affinity chromatography as described previously (14). Carboxyltransferase was isolated from a strain of E. coli that overexpresses the genes coding for the α and β subunits of carboxyltransferase (15). A histidine tag attached to the N terminus of the α subunit allowed for purification using nickel affinity chromatography (14). The histidine tags on both biotin carboxylase and carboxyltransferase were found not to affect the activity of the enzymes. Expression and purification of the BCCP87 protein was according to Chapman-Smith et al. (7). The lyophilized BCCP87 was dissolved in a solution of 10 mM HEPES, pH 7.0, and 100 mM KCl. The concentration of biotin carboxylase, carboxyltransferase, and the BCCP87 protein was determined by the method of Bradford using bovine serum albumin (II) as a standard (16). His-binding resin was from Novagen. Pyruvate kinase was from Roche Molecular Biochemicals. All other reagents were from Sigma or Aldrich.

Enzymatic Assays—The activity of biotin carboxylase was measured by following the production of ADP using the coupling enzymes pyruvate kinase and lactate dehydrogenase as described previously (14). The rate of phosphoryl transfer from carbamyl phosphate to ADP catalyzed by biotin carboxylase was determined spectrophotometrically with the coupling enzymes hexokinase and glucose-6-phosphate dehydrogenase as described by Blanchard and Waldrop (15). Although the number of active sites is not known for carboxyltransferase, we assumed it contained two active sites since the protein is an αβδ tetramer. Values for V and V/K for carboxyltransferase were calculated per active site using a molecular weight of 68,000 for each αβ dimer.

Data Analysis—The parameters Km and Vmax were determined by fitting the initial velocity versus holoBCCP87 concentration curves to the Michaelis-Menten equation using the nonlinear regression program Enzfitter.

RESULTS AND DISCUSSION

The kinetic parameters for the reaction of holoBCCP87 with biotin carboxylase are shown in Table I. The maximal velocity (Vmax) of biotin carboxylase with holoBCCP87 as substrate was 16-fold greater than with biotin as substrate. More significantly, the Michaelis constant (Km) for holoBCCP87 is 530-fold lower than the Km for free biotin. To account for the low Km for holoBCCP87 in terms of the rate of catalysis the V/K parameter or catalytic efficiency of using biotin versus holoBCCP87 as a substrate is compared. HoloBCCP87 is 8000-fold more efficient as a substrate for biotin carboxylase than is biotin. It is highly significant that the Km for holoBCCP87 is much less than the Km for free biotin, because the Km for free biotin has been estimated to be less than 1 μM (17). If the Km for holoBCCP87 was in the millimolar region, as it is for free biotin, the reaction catalyzed by biotin carboxylase would not occur with a rate fast enough to support cell growth. Moreover, the increased Vmax with holoBCCP87 provides strong support for the phenomenon of substrate-induced
synergism exhibited by biotin carboxylase (14). In the absence of biotin, biotin carboxylase will catalyze a bicarbonate-dependent hydrolysis of ATP (18). The $V_{\text{max}}$ of this bicarbonate-dependent ATPase reaction is 0.073 min$^{-1}$ (14). Biotin increased the rate of ATP hydrolysis 860-fold, whereas holoBCCP87 increased ATP hydrolysis almost 14,000-fold. This is very important physiologically because it allows biotin carboxylase to hydrolyze ATP at a significant rate only when the biotin carboxyl carrier protein is present, thereby avoiding a waste of ATP.

Biotin carboxylase is known to catalyze the transfer of a phosphoryl group from carbamyl phosphate to ADP to form ATP and carbamic acid, with carbamyl phosphate believed to act as an analog of the carboxyphosphate intermediate in the reaction normally catalyzed by biotin carboxylase (19). The rate of phosphoryl transfer is stimulated by biotin, which does not participate in the chemistry of the reaction but presumably induces a conformational change that promotes the reaction (19). Given that holoBCCP87 significantly increased the $V_{\text{max}}$ of biotin carboxylase compared with free biotin, it was of interest to determine whether holoBCCP87 stimulated the phosphoryl transfer reaction of biotin carboxylase. The initial velocity of the phosphoryl transfer reaction was measured in the presence of either biotin or holoBCCP87 and compared with the rate in the absence of these components. The data are shown in Fig. 1. The velocity in the presence of 0.05 mM biotin was 3.8-fold greater than in its absence. In contrast, 0.05 mM holoBCCP87 increased the rate 19-fold compared with the control. This was consistent with the observation that the affinity of holoBCCP87 for biotin carboxylase was greater than for free biotin.

The other enzymatic activity of acetyl-CoA carboxylase is carboxyltransferase, which catalyzes the transfer of the carboxyl group from carboxylated biotin to acetyl-CoA to make malonyl-CoA (Scheme 1, Reaction 2). The interaction of holoBCCP87 with carboxyltransferase was assessed in the nonphysiological direction because holoBCCP87 is a product of the reaction and there is a facile spectrophotometric assay for the reverse reaction (15). The $K_{m}$ and $V_{\text{max}}$ values for holoBCCP87 and biotin as substrates for carboxyltransferase in the reverse direction are shown in Table I. The maximal velocity for holoBCCP87 was 140-fold higher than the maximal velocity for biotin, whereas the $K_{m}$ for holoBCCP87 was 15-fold less than the $K_{m}$ for biotin. Thus, the catalytic efficiency of carboxyltransferase with holoBCCP87 as substrate was 2000-fold greater than with biotin as a substrate. Moreover, it is interesting to note that the magnitude of the changes in the $V_{\text{max}}$ and $K_{m}$ values for holoBCCP87 compared with biotin was opposite to that seen for the biotin carboxylase reaction. For biotin carboxylase, the $K_{m}$ for holoBCCP87 was 530-fold lower than it was for biotin, whereas the $V_{\text{max}}$ was only 16-fold higher than with biotin. In contrast, for the carboxyltransferase reaction, the $K_{m}$ for holoBCCP87 reaction was only about 15-fold less than biotin, whereas there was a 141-fold increase in the maximal velocity. The difference in $K_{m}$ values suggests that the protein moiety of holoBCCP87 is less significant in binding to carboxyltransferase than it is in binding to biotin carboxylase.

The detailed molecular mechanism that allowed holoBCCP87 to react faster with biotin carboxylase and carboxyltransferase than does free biotin is not known. A pentapeptide derived from the biotin carboxyl carrier protein and containing the biotinylated lysyl residue was found to be a substrate for biotin carboxylase and the $V/K$ value was the same as for free biotin (20). This suggests that for biotin carboxylase, residues in the biotin carboxyl carrier protein far from the biotinylated lysyl residue are required for the dramatic increase in catalytic efficiency. The lower $K_{m}$ for holoBCCP87 compared with biotin for biotin carboxylase obviously contributed to the higher $V/K$ value for holoBCCP87. However, the tighter binding of BCCP87 did not inhibit the reaction by creating a large energy barrier for reaching the transition state because the maximal velocity for holoBCCP87 as substrate was greater than free biotin. There could be any number of mechanisms by which holoBCCP87 generates an increase in the maximal velocity of biotin carboxylase and carboxyltransferase. Nevertheless, it is worth noting that the results here for biotin carboxylase and carboxyltransferase were somewhat similar to those found for CoA transferase (21, 22). CoA transferase catalyzes the conversion of succinyl-CoA and acetocacetate into succinate and acetoacetyl-CoA. Shorter chain thiols did not accelerate the reaction rate to the same extent as CoA. It was concluded that while the ADP and pantoic acid moieties of CoA were nonreacting portions of the molecule, they were needed for tight binding to the enzyme. The tight binding of CoA to the enzyme then precisely orient the thiol moiety for catalysis. By analogy, most of the protein moiety of holoBCCP87 is far from the site of carboxylation, yet the binding interactions may help to position biotin in the active sites of biotin carboxylase and carboxyltransferase to allow carboxylation to occur more efficiently. The decrease in conformational flexibility of the biotin-binding region of the holoBCCP87 compared with apoBCCP87 is consistent with this hypothesis (13).

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