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Preliminary X-ray Crystallographic Analysis of Biotin Carboxylase Isolated from *Escherichia coli*

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Acetyl CoA carboxylase catalyzes the first committed step in the biosynthesis of long chain fatty acids. In *Escherichia coli*, the enzyme consists of three subunits that are isolated separately and display distinct functional properties. Here we report the crystallization and preliminary X-ray analysis of one of these components, namely biotin carboxylase. The crystals are grown by microdialysis against 10 mM potassium phosphate (pH 7·0), 1 mM EDTA, 2 mM DTT and 1 mM NaN_3 at 4 °C. They belong to the space group $P2_12_12_1$ with unit cell dimensions of $a = 61{\cdot}9$ Å, $b = 96{\cdot}1$ Å and $c = 180{\cdot}6$ Å and contain one dimer per asymmetric unit. The crystals diffract to a nominal resolution of 2·2 Å. From a mechanistic standpoint, biotin carboxylase is especially interesting in that it is the smallest protein within its class and is one of only two carboxylases that can utilize free biotin as a substrate.

Keywords: acetyl CoA carboxylase; biotin carboxylase; crystallization; X-ray analysis; fatty acid synthesis

Acetyl CoA carboxylase catalyzes the carboxylation of acetyl CoA to form malonyl CoA, which constitutes the first committed step in fatty acid biosynthesis (Lane *et al.*, 1974). The enzyme requires biotin for activity and as with other biotin-dependent carboxylases, catalysis is accomplished *via* the two-step reaction mechanism as shown below:

(1) $\text{E-biotin} + HCO_3^- + Mg^{2+}\text{-ATP} \overset{Mg^{+2}}{\rightleftarrows} \text{E-biotin-}CO_2^- + Mg^{2+}\text{-ADP} + P_i$

(2) $\text{E-biotin-}CO_2^- + \text{acetyl CoA} \rightleftarrows \text{E-biotin} + \text{malonyl CoA}.$

The first half reaction, which is ATP-dependent, is carried out by the biotin carboxylase subunit of acetyl CoA carboxylase (Guchhait *et al.*, 1974). The second half reaction, catalyzed by the carboxyltransferase subunit of the acetyl CoA carboxylase complex, involves the transfer of a carboxyl group from biotin to acetyl CoA (Guchhait *et al.*, 1974). A third component, namely the biotin carboxyl carrier protein, contains a biotin molecule covalently attached to Nζ of a lysine residue (Guchhait *et al.*, 1974). It is currently thought that this carrier protein provides a "swinging arm" that moves the biotin moiety between the carboxylase and the carboxyltransferase subunits of the acetyl CoA carboxylase complex (Guchhait *et al.*, 1974). In *Escherichia coli*, these three subunits are isolated separately and display distinct functional properties (Guchhait *et al.*, 1974). In contrast to bacteria and plants, however, the animal acetyl CoA carboxylases exist as stable multisubunit complexes of high molecular mass (Wood & Barden, 1977; Wakil *et al.*, 1983).

The biotin carboxylase subunit represents an ideal system for mechanistic studies on biotin carboxylation in that it retains its enzymatic activity after being isolated from the other subunits of the larger complex. Furthermore, it is one of only two biotin-dependent carboxylases, the other being β-methylcrotonyl CoA carboxylase, that can utilize free biotin as a substrate (Guchhait *et al.*, 1974). Although numerous proposals have been set forth describing the chemical mechanism of biotin carboxylase, all of the evidence to date suggests the following pathway. Initially, Mg^{2+}-ATP serves to activate bicarbonate for nucleophilic substitution through the formation of a carboxyphosphate intermediate (Polakis *et al.*, 1974; Climent & Rubio, 1986; Ogita & Knowles, 1988). The carboxyphosphate intermediate either dissociates to P_i and CO_2 followed by the attack of biotin on CO_2, or

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biotin reacts directly with carboxyphosphate to form a tetrahedral adduct that collapses to form *N*-1-carboxybiotin and inorganic phosphate. The enol (or enolate) tautomer of biotin is considered to be the reactive species, since the keto form of the molecule is a very poor nucleophile (Hegarty *et al.*, 1969; Fry *et al.*, 1985).

In contrast to the basic biochemistry of the reaction, virtually nothing is known about the amino acids located in the active site of the enzyme. We have therefore initiated a structural investigation of the protein by single crystal X-ray diffraction. Biotin carboxylase is especially amenable for such studies in that it is the smallest of the biotin-dependent carboxylases and the gene coding for the enzyme has been recently cloned and overexpressed (Li & Cronan, 1992). Here we report the crystallization and preliminary X-ray analysis of biotin carboxylase as obtained from *E. coli*. It is important to note that while small crystals of the enzyme were first reported nearly 20 years ago, at that time they were simply used as a verification of protein purity and were never crystallographically analyzed (Guchhait *et al.*, 1974).

The enzyme was isolated from the BMH71-18 strain of *E. coli* containing the plasmid pLS101, which carries the genes for both biotin carboxylase and the biotin carboxyl carrier protein under control of the *lac* promoter (Li & Cronan, 1992). Bacteria were grown and biotin carboxylase gene expression was initiated by the addition of isopropylthiogalactoside according to published procedures (Li & Cronan, 1992). The protein was purified according to the procedure of Tipton & Cleland (1988) up to and including the anion exchange chromatography step. However, contrary to previously published purification schemes for biotin carboxylase, the enzyme bound to the DEAE column and was eluted with 1 l of a continuous gradient from 0 to 0·8 M potassium chloride in 10 mM potassium phosphate (pH 7·0) and 1 mM DTT. Fractions containing pure biotin carboxylase were pooled and the enzyme was precipitated by dialysis against 50 mM potassium phosphate (pH 7·0), 1 mM EDTA, 1 mM DTT, 60% (w/v) ammonium sulfate and 15% (v/v) glycerol. The protein was stored as an ammonium sulfate precipitate.

For crystallization experiments, the biotin carboxylase pellets were dissolved in 10 mM Hepes (pH 7·0), 100 mM NaCl, 1 mM DTT and 1 mM NaN_3. Any remaining insoluble protein was removed by centrifugation and the supernatant was dialyzed overnight against the Hepes buffer. Following dialysis the protein was concentrated with a Centricon 30 (Amicon).

Biotin carboxylase crystals were grown at low ionic strength by microdialysis against 10 mM potassium phosphate (pH 7·0), 1 mM EDTA, 2 mM DTT and 1 mM NaN_3 at 4 °C with a protein concentration of 10 mg/ml. These conditions were essentially those employed by Guchhait *et al.* (1974) for the crystallization of non-recombinant biotin carboxylase except that the protein concentration was doubled. Crystals grew as rectangular prisms with typical dimensions of 0·5 mm × 0·5 mm × 1·0 mm and generally appeared within 24 to 48 hours.

For X-ray diffraction experiments, the crystals were mounted in thin-walled quartz capillary tubes (Charles Supper Co.) and air-cooled at 4 °C. Precession photographs were recorded with nickel-filtered copper K_α radiation from a Rigaku RU200 rotating anode X-ray generator with a 200 μm focal spot and operated at 50 kV and 50 mA. Precession photographs of the *h0l* and the *hk*0 zones demonstrated that the crystals belonged to the orthorhombic space group $P2_12_12_1$ with unit cell dimensions of $a = 61{\cdot}9$ Å, $b = 96{\cdot}1$ Å and $c = 180{\cdot}6$ Å. Assuming a molecular mass of 98,792 daltons (Li & Cronan, 1992) and one dimer per asymmetric unit, the V_m value was 2·72 Å^3/Da, which lies within the normal range (1·68 to 3·53 Å^3/Da) observed for globular proteins (Matthews, 1968). On the basis of a preliminary native data set, collected from a single crystal with a Siemens X-1000 area detector and processed with the XDS data reduction software package (Kabsch, 1988*a*,*b*), the crystals diffract to a nominal resolution of 2·2 Å. Overall, 93,957 reflections were collected for this X-ray data set and were reduced to 41,566 independent reflections with an R_{sym} of 4·0%, where $R_{sym} = \Sigma|I - \bar{I}|/\Sigma I$. The R_{sym} for the highest resolution shell of 2·2 Å was 21·9% for all measured X-ray data. A Patterson self-rotation function search with X-ray data from 9·0 Å to 3·5 Å resolution yielded a peak at 4·5σ, thereby suggesting the orientation of the local dyad for the dimeric enzyme (Tong & Rossmann, 1990). Thus far, four heavy atom derivatives have been prepared by soaking crystals in $LuCl_3 \cdot 6H_2O$, triethyllead acetate, K_2IrCl_6 and $KAu(CN)_2$. The heavy atom binding positions for these derivatives have been located by inspection of appropriate Patterson maps. These derivatives, while isomorphous, are binding at special positions, however. Consequently, a search for other potential heavy atom derivatives is presently underway.

The three-dimensional structure of biotin carboxylase will yield considerable insight into its catalytic mechanism and should facilitate answers to several questions. For example, how does the enzyme stabilize carboxyphosphate, a relatively unstable molecule with a half-life of 70 milliseconds (Sauers *et al.*, 1975)? How is biotin converted from the keto to the enol form by the enzyme? Answers to these questions will not only aid in mechanistic studies of other biotin-dependent carboxylases but will also contribute to understanding the mechanisms of carbamyl phosphate synthetase and phosphoenolpyruvate carboxylase. The amino acid sequence of carbamyl phosphate synthetase from *E. coli* is strikingly similar to that of biotin carboxylase (Li & Cronan, 1992), and like the biotin-dependent carboxylases, carbamyl phosphate synthetase employs Mg^{2+}-ATP in its reaction with bicarbonate

to form a carboxyphosphate intermediate (Wimmer *et al.*, 1979). Likewise, the reaction catalyzed by phosphoenolpyruvate carboxylase is also thought to proceed *via* a carboxyphosphate intermediate (O'Leary *et al.*, 1981).

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