*TRANSCARBOXYLASE, VIII. ISOLATION AND PROPERTIES OF A BIOTIN-CARBOXYL CARRIER PROTEIN**

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Abstract.—Methylmalonyl CoA-oxalacetate transcarboxylase (EC 2. 1. 3. 1) from *Propionibacterium shermanii* is a biotin enzyme of 670,000 molecular weight containing 6 moles of biotin per mole of enzyme. The active enzyme dissociates spontaneously at low ionic strength and alkaline pH to a mixture of inactive subunits. One type of subunit contains all the biotin of the original molecule. The biotin unit has an $s_{20,w} = 1.3S$ and a molecular weight of approximately 12,000. It contains 1 mole of biotin and 1 half-cystine per mole. Qualitative dansyl techniques indicate that alanine is the amino terminal residue of the biotin subunit.

Transcarboxylase is a unique biotin-containing enzyme occurring in propionic acid bacteria which catalyzes the reversible transfer of a carboxyl group from methylmalonyl CoA to pyruvate, yielding propionyl CoA and oxalacetate.[1, 2] It differs from the other biotin enzymes which catalyze the utilization of CO_2 in that free CO_2 is not involved and neither ATP nor a divalent metal is required. The biotin is covalently linked to the enzyme as an amide of the ϵ amino group of lysine[3] and the carboxyl is transferred to the 1′-N of the biotin[3] giving a carboxylated intermediate similar to those formed by enzymes which catalyze CO_2 fixation. Ultracentrifugation of transcarboxylase gives two components with sedimentation coefficients of 18*S* and 16*S* at ~60,000 or ~50,000 rpm; however, at ~40,000 or ~30,000 rpm only a single component appears with an $s_{20,w} = 17S$.[4] Transcarboxylase dissociates spontaneously at low ionic strength and alkaline pH to inactive subunits with an $s_{20,w} = 6S$ and there is an intermediate species of 12*S*.[4, 5] The 6*S* component is made up of two species, one of which contains the biotin and the other the metals, Co and Zn.[2, 5, 6] There are a total of 6 moles of Co plus Zn per mole of transcarboxylase[2, 5] and these are tightly bound to the 6*S* subunit.[5] Acidification of the dissociated inactive enzyme results in reassociation to an active enzyme with an $s_{20,w} = \sim 24S$.[4]

We wish to report here the isolation of a subunit which arises from the 6*S* biotin subunit and has an $s_{20,w}$ value of 1.3*S*. It contains essentially all the biotin found in the original enzyme. A preliminary report[7] of these findings has been previously communicated.

Materials and Methods.—Biotin was obtained from Calbiochem and tritiated by New England Nuclear. Avidin was from Worthington and 2,4′-hydroxybenzeneazobenzoic acid from Sigma. Sepharose 2B, DEAE Sephadex, and all grades of Sephadex were obtained from Pharmacia while the desalting resin (AG 11A8 50–100 mesh) was purchased from Bio-Rad. Trizma base was the purest grade available from Sigma. All other enzymes and reagents were the best grades available commercially.

Transcarboxylase was determined as described by Wood *et al.*[2] in a coupled spectrophotometric assay in which the oxalacetate is reduced with DPNH using malate dehydro-

genase. *Propionibacterium shermanii* was grown on a medium containing H-biotin and the labeled transcarboxylase was isolated as described previously.[1-3] It had a specific activity of 40 μmoles of oxalacetate formed per minute per milligram of protein. The protein was determined spectrophotometrically using the formula of Kalckar as suggested by Lowry[8] and by a microbiuret method[9] with bovine serum albumin as the standard. Tritium was measured with 25% efficiency in a dioxane-based scintillant[10] on a Nuclear-Chicago Mark I liquid scintillation counter equipped with an external standard. Corrections for unequal quenching were made when the variation was greater than $\pm 5\%$.

The biotin-carboxyl carrier protein was isolated using a column of DEAE Sephadex at pH 8.8 as described in *Results*. It occurred in the breakthrough fractions and the eluate with 0.1 *M* KCl (Fig. 3). The protein was prepared for analysis by lyophilizing the solution to dryness and redissolving it in a minimal volume of water which was desalted at room temperature in 3-ml aliquots over a column (2.0 × 50 cm) of AG 11A8 50–100 mesh equilibrated with water. The protein was traced by radioactivity and the salt was traced by turbidity with $AgNO_3$. Samples were pooled and the procedures were repeated until the salt and radioactivity were separated. The pooled lyophilized sample was then transferred to 0.1 *M* NaCl adjusted to pH 8.3 by passage over a column (1.2 × 90 cm) of Sephadex G-10.

Polyacrylamide gel electrophoresis was done at pH 9.0 by the method of Davis.[11] Protein was visualized in the gel by denaturation for 2 hr in 10% trichloroacetic acid, followed by staining for at least 4 hr in 0.1% Coomassie brilliant blue in 7.5% acetic acid. Gels were destained rapidly by placing them in pierced dialysis sacs which were suspended in 7.5% acetic acid and stirred magnetically.

Ultracentrifugation was performed on a Spinco model E ultracentrifuge equipped with Schlieren and interference optics. The molecular weight was determined by the Yphantis techniques[12] in a standard 12-mm double sector cell containing 60 μg of biotin-carboxyl carrier protein in 100 μl. Two speeds (44,000 and 52,000 rpm) were used and two independent calculations of Rayleigh interference patterns were made at each speed. The determined molecular weight is the mean of the four values. The sucrose gradient centrifugation was done with a linear 5 to 20% concentration of sucrose according to the method of Martin and Ames[13] on a Spinco L2 65 ultracentrifuge with an SW-56 rotor at 50,000 rpm. Fractions were collected from the bottom of each tube without pressure.

Amino acids were determined on a Spinco model 120B which had been adapted to the accelerated technique and expanded scale from 0 to 0.10 μmole of amino acid.[14, 15] Hydrolysis for 20 hr was performed in evacuated tubes using 6 *N* HCl. Performic acid oxidation was performed according to the method of Moore.[16]

Biotin was determined on a pronase digest of transcarboxylase by the micro method of Green.[17] Biotin standards were subjected to the same treatment as enzyme samples. The standard varied from 0 to 1.5 μg of biotin in 0.25 μg increments. Two samples, 30 and 48 μg of protein, were assayed. All samples were heated at 60° for 10 min and then cooled to room temperature; pronase was added and the mixture was then incubated at 25° for 16 hr. All digestions were in 200 μl of 50 mM phosphate buffer, pH 6.8, containing 30 μg of pronase. After digestion, 200 μl of 0.1 *M* phosphate buffer, pH 6.5, was added followed by 150 μl of a mixture containing 120 μg avidin and 29 μg 2,4′-hydroxybenzeneazobenzoic acid in the 0.1 *M* phosphate buffer. Absorbance at 500 mμ was then determined and the biotin in the samples was determined by comparison with the standard. Some insoluble peptides which were formed by pronase digestion were removed by centrifugation before development of the color. The distribution of tritium in the supernatant solution and precipitate was determined (approximately 85% in solution) and a correction was made on this basis for insoluble biotin peptides. The values were significantly lower in determinations which were performed without pronase digestion.

N-terminal amino acid analysis was performed by the dansyl technique as described by Gros and Labouesse.[18] The thin-layer systems used to separate basic amino acids (system 86) and neutral and acidic (system 85) amino acids were those described by Pataki.[19] High-voltage electrophoresis was performed on a Shandon flat plate instrument with a cooled bed as described by Gray.[20]

Results.—Dissociation: When transcarboxylase ($s_{20,w}$ = 16,18*S*) is transferred at 4° to 0.05 μ Tris-HCl,pH 8.8,[21] a rapid loss of enzymatic activity occurs and there is dissociation to subunits with sedimentation coefficients of 12*S* and 6*S*.[4] After 72 hr, only one component with a sedimentation coefficient of 6*S* was evident as shown in Figure 1. The 1.3*S* component is not visible because of its low concentration and rapid diffusion.

When the dissociated preparations were analyzed by sucrose gradient centrifugation in 0.05 μ Tris-HCl, pH 8.8, the dissociated material showed two components (Fig. 2). One component comprised the bulk of the protein and another slower-sedimenting component contained all of the biotin. Transcarboxylase containing tritiated biotin was used throughout these studies so that the position of the biotin was always marked.

Ion-exchange chromatography: Dissociated transcarboxylase was applied at 4° to a column of DEAE Sephadex A-50 equilibrated with 0.05 μ Tris-HCl, pH 8.8, and eluted stepwise by increasing concentrations of KCl (Fig. 3). Recovery of protein and radioactivity from this column was quantitative. Sedimentation velocity centrifugation of the fractions obtained with 0.2 *M* and 0.8 *M* KCl gave $s_2{}^0{}_{,w}$ values of 6*S* and 12*S*, respectively (Fig. 4). It is noted (Fig. 3) that the 12*S* component does not contain biotin. No appreciable enzymatic activity was detected in the eluates from the column. The small breakthrough peak and the 0.1 *M* KCl eluate contained practically all the biotin. This fraction was prepared for analysis as described in *Materials and Methods*. A portion of the resulting material was concentrated to 2.0 mg per milliliter by vacuum dialysis and

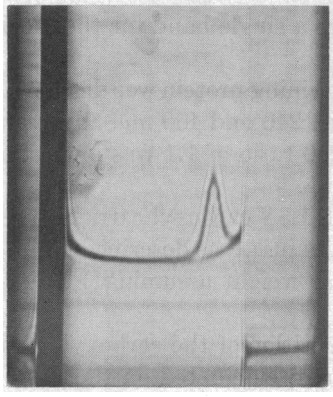

FIG. 1.—Sedimentation patterns from dissociated transcarboxylase centrifuged in a 12-mm double sector cell in a Spinco model E ultracentrifuge at 60,000 rpm at 4.6°. Photographs were taken after 24 min at a phase angle of 45°. The protein concentration was 3.5 mg/ml in 0.05 μ Tris-HCl, pH 8.8, at 4°. The enzyme had been held for 75 hr in this buffer prior to the centrifugation. The $s_{20,w}$ = 6 *S*.

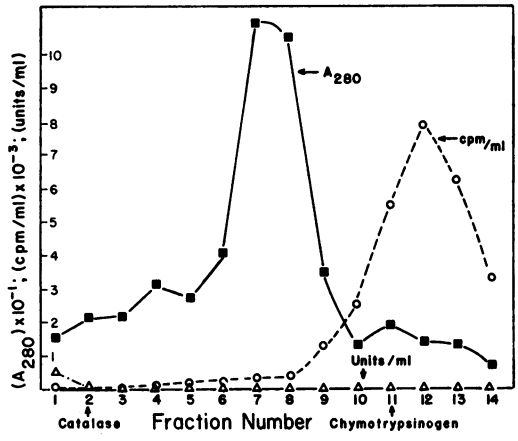

FIG. 2.—Sucrose density gradient centrifugation of dissociated transcarboxylase. Dissociated transcarboxylase (3.1 mg, spec. act. 0.12) in 0.2 ml, 0.05 μ Tris-HCl, pH 9.0, was layered over 4 ml of a 5–20% sucrose gradient containing 0.05 μ Tris-HCl buffer, pH 9.0, and centrifuged at 50,000 rpm for 15 hr at 4°. Fractions were collected from the bottom. Aliquots were followed at 280 mμ (■——■), radioactivity was measured (○- -○), and the transcarboxylase activity was assayed (△- -△) as described in *Materials and Methods*.

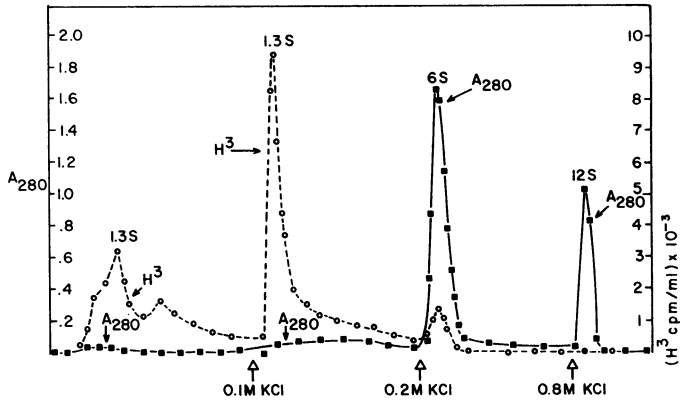

FIG. 3.—Chromatography of dissociated transcarboxylase on DEAE Sephadex. Thirty-nine ml of solution containing 73.5 mg of dissociated transcarboxylase (spec. act. 0.007) labeled with 830,000 cpm as tritiated biotin was applied to a column (1.2 × 40 cm) of DEAE Sephadex A-50 equilibrated with 0.05 μ Tris-HCl, pH 8.8, at 4°. The protein had previously been transferred to the same buffer by passage over Sephadex G-25 at 4° and allowed to dissociate. For elution the concentration of KCl in the Tris-HCl buffer was increased stepwise. The 0.1 *M* KCl was started at 287 ml, the 0.2 *M* at 539 ml, and 0.8 *M* at 763 ml. The column was washed with 0.1 *M* NaOH after 966 ml had been collected. The protein was monitored by A_{280} (■——■) and the radioactivity (O- - -O) as described in *Materials and Methods*.

sedimented in a synthetic boundary cell on the analytical ultracentrifuge (Fig. 5). A sharp, rapidly diffusing boundary was observed with a sedimentation coefficient of 1.3*S*.

Polyacrylamide gel electrophoresis at pH 9 gave a single band for the biotin-containing unit.

Spectrum: The spectrum of the 1.3*S* biotin containing protein was determined on a Cary 14 recording spectrophotometer between 240 and 400 mμ. It has no significant absorption above 320 mμ. The 280:260 ratio of 1.1 was lower than observed in most protein and the λ_{max} was at 274 mμ.

Molecular weight: The molecular weight of the 1.3*S* carboxyl carrier protein was determined by the "high speed" method of Yphantis,[12] as described in *Materials and Methods*. The mean value for molecular weight assuming $\bar{V} = 0.75$ was $11.7 \times 10^3 \pm 1 \times 10^3$.

Amino acid composition: The amino acid composition of the carboxyl carrier protein is shown in Table 1. The molecular weight, calculated on the basis of 1 half-cystine residue per mole, is 11.0×10^3. A spectrophotometric method[22] for tryptophan indicated there was no tryptophan present. This observation needs further confirmation.

Biotin content: Two determinations on the protein gave values of 0.89 and 0.74 moles of biotin per mole of protein based on a molecular weight of 12×10^3 for the protein. These values were corrected for insoluble biotin peptides. Although the values vary considerably and are well below 1.0, it seems likely that availability of more material and improved techniques will increase the accuracy.

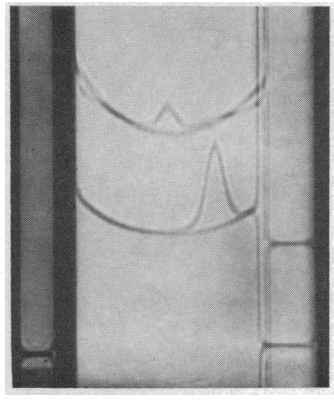

FIG. 4.—Sedimentation Schlieren patterns of fractions obtained from the experiment of Fig. 3. The peak tubes of 0.2 *M* KCl (lower pattern = standard cell) and 0.8 *M* KCl (upper pattern = wedge cell) were centrifuged in a Spinco model E ultracentrifuge at 50,740 rpm at 3.83°. The runs were made in the eluting buffers. The photograph was taken after 64 min at a bar angle of 55°. The protein concentrations were 1.90 mg/ml for the 0.2 *M* KCl and 1.06 mg/ml for the 0.8 *M* KCl fractions. The $s_{20,w}$ values are 6*S* and 12*S*, respectively.

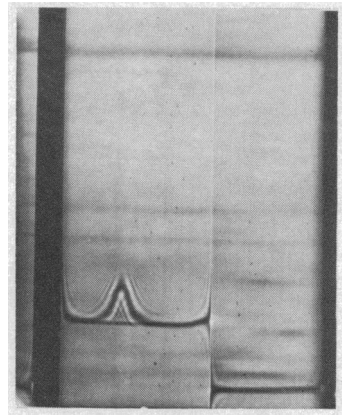

FIG. 5.—Sedimentation Schlieren patterns in a Spinco model E ultracentrifuge at 59,780 rpm at 4.31° of biotin-rich fractions from DEAE Sephadex column of Fig. 3. The sample (2 mg/ml in 0.1 *M* NaCl, adjusted to pH 8.3) was in a 12-mm synthetic boundary cell and 0.1 *M* NaCl was layered over it by centrifugal force. The photograph was taken after 8 min at a bar angle of 55°. The $s_{20,w} = 1.3S$.

N-terminal amino acid: The protein (0.2 mg) was subjected to dansylation, trichloroacetic acid precipitation, and a 4-hr hydrolysis as described by Gros and Labouesse.[18] The resultant material was chromatographed on thin-layer plates and two dansylated products were identified. The major product was ϵ-dansyllysine as would be expected for a molecule with eight lysine residues. The only other spot was identified as dansylalanine. This was done by thin-layer chromatography (as described in *Materials and Methods*), simultaneously with standards for the dansylated products of phenylalanine, tryptophan, methionine, and proline, as well as alanine. A strong spot corresponded precisely with dansylalanine. All other fluorescent material remained at the origin or moved as dansylhydroxide or dansylamine. The spot at the origin was shown to be ϵ-dansyllysine by chromatography in a system[19] designed to resolve the dansylated products of basic amino acids and by free electrophoresis in eight per cent formic acid.[20]

Discussion.—Methylmalonyl CoA-oxalacetic transcarboxylase is an enzyme of 670,000 molecular weight containing 6 moles of biotin and 6-g atoms of metal per mole of enzyme.[2, 5] The enzyme dissociates readily at mildly alkaline pH values and low ionic strength with a concomitant loss of enzymatic activity.[4] The metals are present in a 6*S* subunit,[5, 6] and the biotin-carboxyl carrier protein dissociates from a second 6*S* subunit[6] and has a sedimentation coefficient of 1.3*S*.

The properties of this small biotin protein are of obvious interest. It has a molecular weight of 12,000, an amino terminal alanine residue, and 1 mole of bio-

TABLE 1. *Amino acid composition of biotin carboxyl carrier protein.*

Amino acid	Molar ratios	Residues/mole
Lysine	7.50	8
Histidine	2.05	2
Arginine	2.21	2
Aspartic acid	8.27	8
Threonine	5.29	5
Serine	4.42	4
Glutamic acid	8.04	8
Proline	11.56	12
Glycine	10.70	11
Alanine	9.06	9
Half cystine*	1.00	1
Valine	8.75	9
Methionine	2.19	2
Isoleucine	4.58	5
Leucine	5.98	6
Tyrosine	1.95	2
Phenylalanine	2.98	3
Total		97

* Half cystine, determined on a performic acid oxidized sample, gave the lowest value for μmoles and this was used as 1.00 for the determination of ratios of amino acids.

tin per mole of protein. Close agreement is obtained between molecular weight values deduced from ultracentrifugal data and from amino acid analysis. The presence of eight lysine residues per mole insures ample sites for the convalent linkage of the biotin molecule. The amino acid composition of the protein is not remarkable except for the presence of a single half-cystine residue per mole and the apparent absence of tryptophan. There is a rather high concentration of proline. Transcarboxylase has not shown sensitivity to *S*H reagents;[1] this group may be unimportant or structurally masked in the intact protein.

The obvious similarities between this biotin-carboxyl carrier protein and the acyl carrier protein, lead to speculation that it has evolved as a biotin and, therefore, an activated CO_2 carrying unit. The recent report of Alberts *et al.*[23] indicates that a similar biotin unit exists in acetyl CoA carboxylase of *E. coli*. These findings, together with those reported above, strengthen the possibility that a similar biotin-carboxyl carrier protein may be found in the structures of other biotin enzymes. It is important to note that transcarboxylase is the only biotin enzyme known which catalyzes the transfer of carboxyl groups between organic compounds. Activation energy in the form of ATP is not required and the reaction thus differs from that catalyzed by other biotin enzymes which catalyze fixation of CO_2. The latter are the more biologically prevalent and involve activation by ATP, addition of HCO_3^- to biotin, and transfer to an acceptor. It will be of interest to determine whether biotin-carboxyl carrier proteins obtained from different enzyme may be used interchangeably. If this occurs, it will show that the other components of the enzyme specify the reaction to be catalyzed and that the carboxyl carrier protein is a passive acceptor and donor of "active carboxyl" units with no substrate binding sites.

Carboxylation of free biotin has been observed with β-methyl crotonyl CoA carboxylase[24] and acetyl CoA carboxylase.[25] It has generally been considered

that there are sites for binding the biotin ring which are located adjacent to the substrate sites and, because of the length of the lysine and valeric acid side chain, there is sufficient flexibility to permit migration of the biotin ring between the different substrate sites.[24] At high concentrations, the free biotin apparently competes with the covalently linked biotin of the enzyme, binds at a biotin site, and is carboxylated. Alberts *et al.*[23] have observed carboxylation of free biotin with ATP and CO_2 which is catalyzed by a subunit obtained from acetyl CoA carboxylase. This carboxylation occurs in the absence of the biotin-carboxyl carrier protein. Thus, there is direct evidence that the subunit specifies the activation of the CO_2 by ATP and that there is a binding site for biotin on the nonbiotin subunit. Although similar carboxylation of free biotin has not been observed with intact transcarboxylase or with its subunits, evidence has been obtained by study of the kinetics of the inhibition of exchange reactions that there are sites which bind the biotin ring adjacent to the substrate binding sites. The two substrate sites and the respective biotin sites for the ring appear to be located on separate subunits of transcarboxylase[26] and the carboxyl carrier protein thus serves to transfer the carboxylated biotin between these sites.

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