Isolation of Peptides from the Carboxyl Carrier Subunit of Transcarboxylase. Role of the Non-Biotinyl Peptide in Assembly†

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ABSTRACT: Transcarboxylase is made up of a central hexameric subunit ($s_{20,w}$ ~12 S), three peripheral dimeric metallo subunits ($s_{20,w}$ ~5 S), and six biotinyl carboxyl carrier subunits ($s_{20,w}$ ~1.3 S). The results presented here show that the carboxyl carrier subunit is required for assembly of the 12S and 5S subunits into the oligomer. However, only a portion of the subunit is required for this assembly. On treatment of transcarboxylase briefly with trypsin at pH 6.3 extremely susceptible peptide bonds of the carboxyl carrier protein are cleaved releasing biotinyl peptides of about ~66 and ~40 residues. The resulting trypsinized transcarboxylase, though enzymatically inactive, remains essentially intact as judged by its hydrodynamic and molecular sieving properties. The modified enzyme can be dissociated at pH 8 to the central 12S subunit and peripheral 5S subunit to which the residual portion(s) of the cleaved carboxyl carrier protein is still attached. These components can then be separated by molecular sieving. The residual portion of the carboxyl carrier protein (non-biotinyl peptide) can then be isolated by dissociation of the 5S subunit complex at pH 9 and by chromatography over Bio-Gel A-1.5m. The isolated non-biotinyl peptide has been shown to contain the combining domain of the $1.3S_E$ carboxyl carrier protein since it causes combination of the 12S and 5S subunits. Active enzyme is formed by combination of the intact carboxyl carrier protein and the 12S and 5S subunits and an inactive oligomer of similar size is formed if the non-biotinyl peptide is used in place of the carboxyl carrier protein. The ~66- and ~40-residue biotinyl peptides, which are released by the trypsin treatment, apparently occur on an exposed portion of the enzyme. This portion of the carboxyl carrier protein apparently serves to place the biotinyl group adjacent to the two substrate sites of the enzyme, one of which is on the peripheral subunit and the other on the central subunit. Thus the carboxyl carrier protein has two functions: one portion holds the 12S and 5S subunits in juxtaposition and the other portion orients the biotinyl group adjacent to the substrate sites so that it may function as a carboxyl carrier between the sites.

Oxalacetate transcarboxylase (EC 2.1.3.1) is a biotinyl enzyme from propionibacteria which catalyzes the following reaction:

$$CH_3{-}\underset{|}{\overset{COO^-}{CH}}{-}COSCoA + CH_3{-}CO{-}COO^- \rightleftharpoons CH_3{-}CH_2{-}COSCoA + {}^-OOC{-}CH_2{-}CO{-}COO^-$$

The polymeric enzyme of molecular weight 790,000 consists of three types of subunits, a central 12S subunit, three peripheral 5S subunits, and six biotinyl carboxyl carrier subunits. There is a considerable body of evidence that these subunits are arranged as diagrammed in Figure 1 (Green, 1972; Green et al., 1972; Wood, 1972; Wood et al., 1973).

The present study describes an investigation of the structure of transcarboxylase by treatment with trypsin. The results show that certain peptide bonds of the biotinyl carboxyl carrier subunit are readily cleaved by trypsin with the formation of two biotinyl peptides of ~40 and ~66 residues and that the remainder of the enzyme remains essentially intact. Evidence is presented that the portion of the biotinyl carboxyl carrier subunit which is not accessible to the trypsin serves to bind the $5S_E$ subunits to the central $12S_H$ subunits as shown in Figure 1.

Experimental Section

Transcarboxylase was isolated from *Propionibacterium shermanii* and assayed as described by Wood et al. (1969). The enzyme had a specific activity of about 40 IU on isolation but at times lost some activity on storage. The biotinyl group of the transcarboxylase was usually labeled with ^3H by growing the bacteria in medium containing [^3H]biotin. The reagents were as previously described (Jacobson et al., 1970; Wood et al., 1969).

Dissociation of the enzyme, isolation of the subunits, and reconstitution of the enzyme from subunits were done as have been described (Ahmad et al., 1970; Jacobson et al., 1970; Wood et al., 1975). Biotin was determined in the isolated enzyme or subunit by a modified colorimetric method (Gerwin et al., 1969) of Green (1970) and the specific radioactivity (cpm/nmol) was calculated on the basis of the radioactivity of the sample. For determination of the amino acid composition, the peptides were concentrated by rotary evaporation in the presence of a drop of octanol and then transferred to 10% acetic acid over a Sephadex G-25 column previously equilibrated with the solvent. Acetic acid was removed by repeated evaporations and the material ob-

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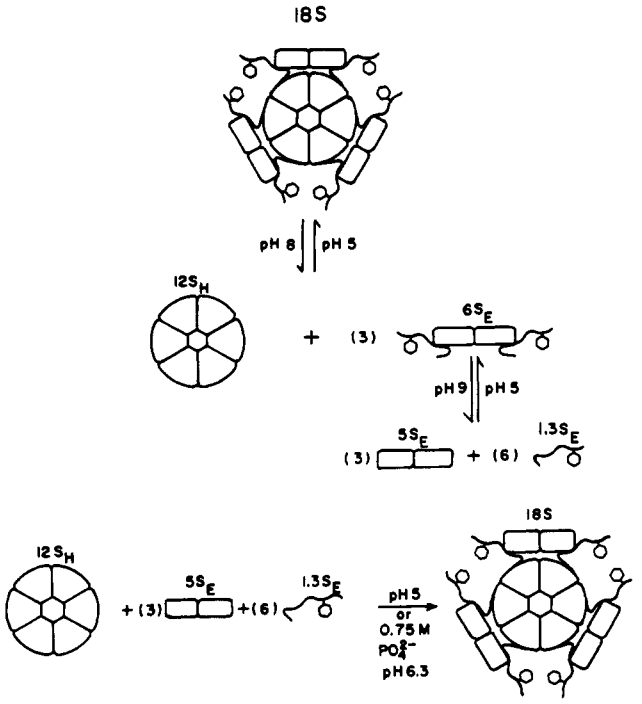

FIGURE 1: Diagrammatic representation of the proposed structure of transcarboxylase. The center structure of molecular weight 360,000 and $s_{20,w}$ ~12 S is designated as the $12S_H$ subunit and is made up of six peptides. The three peripheral subunits of molecular weight 120,000 and $s_{20,w}$ ~5 S are designated as $5S_E$ subunits and each is made up of two peptides. Each of the three peripheral subunits are linked to the central $12S_H$ subunit by two subunits of molecular weight 12,000 and $s_{20,w}$ ~1.3 S which are designated $1.3S_E$ subunits or the biotinyl carboxyl carrier proteins. The biotinyl group is indicated by the hexagon linked to the peptide chain of the $1.3S_E$ subunit. Transcarboxylase dissociates at pH 8 to the $12S_H$ central subunit and to the peripheral $5S_E$ subunits with two $1.3S_E$ subunits still attached to each $5S_E$ subunit. The latter has a sedimentation coefficient of ~6 S and is designated as the $6S_E$ subunit. The $6S_E$ subunit dissociates at pH 9 to the $5S_E$ subunit and to the $1.3S_E$ subunits. The $12S_H$ subunit likewise, dissociates at pH 9 yielding three dimers which are designated $6S_H$ and have a molecular weight 120,000 and $s_{20,w}$ ~6 S. The subscripts H and E have been used because in the electron microscope the enzyme with two peripheral subunits resembles a profile of Mickey Mouse with the central subunit being the head and the peripheral subunits the ears. The subunits recombine to the active enzyme at pH 5 or in a high concentration of phosphate buffer at pH 6.3.

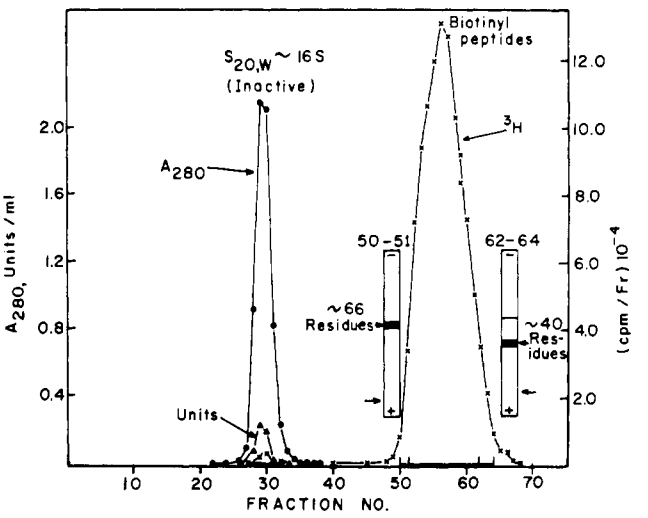

FIGURE 2: Separation of trypsinized enzyme (fractions 27-32) from the resulting ~66 residue and ~40 residue biotinyl peptides (fractions 50-64) by chromatography on Sephadex G-50. Fractions 50-51, 52-61, and 62-64 were pooled separately. Fractions 50-51 contained the ~66-residue peptide and fractions 62-64 the ~40-residue peptide (see Table I). The inserts are diagrams from the polyacrylamide gel electrophoresis of these fractions. The trypsin treatment was conducted as follows: TPCK-trypsin (Worthington Biochemical Corp.) (1.6 mg) dissolved in 0.32 ml of 0.001 *N* HCl was added to the transcarboxylase (163 mg, specific activity = 43 and containing ^{3}H-labeled biotin, 1300 cpm/nmol) in 5 ml of 0.2 *M* potassium phosphate (pH 6.3). After 5 min at room temperature, 0.2 ml of 2% Dip-F was added with stirring and the solution was cooled to 0°. After 5 min, an additional 0.2 ml of Dip-F was added and the solution was allowed to stand for 10 min. A portion of the solution was assayed for transcarboxylase activity and it had a specific activity of <0.1, a loss of more than 99% of the activity. The solution was applied to a 4.4 × 110 cm column of Sephadex G-50-fine at 4° equilibrated with 0.2 *M* phosphate buffer (pH 6.3) containing 0.02% sodium azide and was eluted with the same buffer. Fractions of ~17 ml were collected and the absorption at 280 mμ (●), enzymatic activity (▲), and radioactivity (x) were determined. Fractions 27-33 were combined and precipitated by addition of ammonium sulfate to 80% saturation. Fractions 50-51, 52-61, and 62-64 were separately pooled and concentrated on a rotary evaporator, desalted over a Sephadex G-25 column, and then concentrated again. A portion of pool 50-51 and 62-64 was subjected to gel electrophoresis at 4 mA/gel. The gels contained 7.5% acrylamide and Tris-glycine buffer at pH 8.3 was used (Jovin et al., 1964). The remaining portions were hydrolyzed with hydrochloric acid for amino acid analysis (see Table I).

tained was hydrolyzed with constant boiling HCl. The hydrolysates were analyzed on a Beckman 120 B or Durrum amino acid analyzer. Other methods are indicated in the legends to the tables or figures.

Results

Cleavage of Biotinyl Peptides from Transcarboxylase by Mild Treatment with Trypsin and Isolation of the Products. These studies were initiated because it had been proposed (Green et al., 1972; Wood et al., 1973) that the peripheral $5S_E$ subunits of transcarboxylase are linked to the central subunit by an extended peptide chain and it seemed likely that an exposed portion of this chain might be cleaved by trypsin leaving the residual enzyme or subunits intact. Since transcarboxylase is stable in high concentrations of phosphate and at a somewhat acid pH, the trypsin treatment was done in 0.2 *M* phosphate at pH 6.3. It was found that there was an extremely rapid inactivation of the transcarboxylase and if the action of trypsin was terminated by the addition of Dip-F[1] as soon as the activity was low, there was very little change in the sedimentation pattern of the product from that of the untreated transcarboxylase.

Results are shown in Figure 2 in which transcarboxylase with the biotinyl group labeled with ^{3}H was treated with trypsin and then chromatographed on Sephadex G-50. The major portion of the protein containing only a trace of the original enzyme activity and radioactivity was recovered in one peak and the radioactivity occurred in a second well-separated peak. The protein peak (fraction 27-33) was pooled and precipitated with ammonium sulfate. The ra-

[1] Abbreviations used are: Dip-F, diisopropyl fluorophosphate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate, TC, transcarboxylase, T-TC, trypsinized transcarboxylase from which the resulting biotinyl peptides have been separated (Figure 2). $6S_E$(T-TC) and $1.3S_E$(T-TC) indicate the subunits isolated from the trypsinized transcarboxylase. $1.3S_E$(T-TC) also is designated as the non-biotinyl peptide(s). The $12S_H$ and $5S_E$ subunits may be modified somewhat by the trypsin but for brevity we have only occasionally used the designation $12S_H$(T-TC) and $5S_E$(T-TC) when these designations were needed for clarity.

Table I: Amino Acid Composition of Biotinyl Peptides Resulting from Mild Treatment of Transcarboxylase with Trypsin.[a]

Amino Acid	Pool 50–51 (Figure 2) Molar Ratio	Pool 50–51 (Figure 2) Residues per mole	Pool 62–64 (Figure 2) Molar Ratio	Pool 62–64 (Figure 2) Residues per mole
Lysine	6.53	7	3.38	3
Arginine[b]	1.00	1	1.00	1
Aspartate	4.33	4	3.26	3
Threonine	4.13	4	2.74	3
Serine	1.47	1	1.31	1
Glutamate	9.33	9	5.91	6
Proline	2.87	3	2.08	2
Glycine	11.20	11	5.09	5
Alanine	8.40	8	3.94	4
Valine	7.80	8	4.62	5
Methionine	1.33	1	0.86	1
Isoleucine	3.47	3	1.85	2
Leucine	4.67	5	2.69	3
Phenylalanine	0.53	1	0.51	1
Total residues		66		40

[a] Hydrolysis for 23 hr was performed in evacuated tubes using 6 *N* HCl; 0.05 μmol of norleucine was added as an internal standard and the samples were dried under nitrogen and then dissolved in citrate buffer (pH 2.2). The amino acids were analyzed as described in the Experimental Section. [b] Arginine was taken as the lowest common denominator since phenylalanine would give a value equal or greater than the entire $1.3S_E$ subunit.

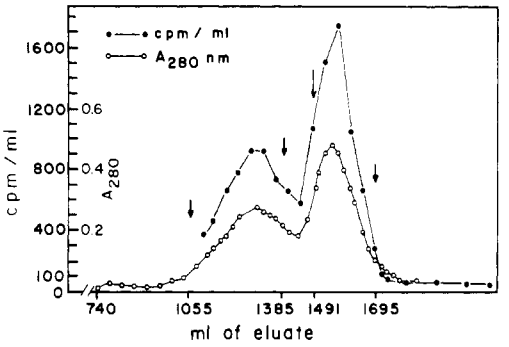

FIGURE 3: Dissociation at pH 8 of trypsinized transcarboxylase labeled with [^{3}H]lysine and isolation of the $6S_E$(T-TC) subunit (1491–1691 ml) by chromatography on Bio-Gel A-1.5m. The sedimentation profiles of the fractions are shown in Figure 4. Transcarboxylase of specific activity ~40 was isolated from propionic acid bacteria grown in media containing [^{3}H]lysine and treated with trypsin and the resulting biotinyl peptides were separated from the trypsinized protein (T-TC) as described in the experiment of Figure 2. The ammonium sulfate precipitate of T-TC was suspended in 0.1 *M* phosphate buffer to a total volume of 6.7 ml containing ~270 mg of protein and was dialyzed for 18 hr at 4° against 2 l. of 0.05 *M* Tris-sulfate (pH 8.0) containing 20% glycerol and 10^{-4} *M* $PhCH_2SO_2F$ and another 6 hr with a change of buffer. The product was placed on a 4.4 × 160 cm column of Bio-Gel A-1.5m (100–200 mesh) which had been equilibrated with 0.05 *M* phosphate buffer (pH 7.0) containing 10^{-4} *M* $PhCH_2SO_2F$ and was eluted with the same buffer. The fractions were assayed for absorbance (○) at 280 nm and for radioactivity (●). The eluates from 1055 to 1385 ml, from 1385 to 1491 ml, and from 1491 to 1695 ml were each precipitated with ammonium sulfate to 80% saturation. Solutions of the proteins were each dialyzed against 500 ml of 0.1 *M* phosphate buffer (pH 6.8). The recovery of protein from the 1055 to 1385 ml pool was ~63 mg, from the 1385 to 1491 ml pool, ~24 mg, and from the 1491 to 1695 ml pool, ~70 mg.

dioactive fractions 50–51, 52–61, and 62–64 were separately pooled, concentrated, desalted, and concentrated again. Polyacrylamide gel electrophoresis of a portion of the pool of fraction 50–51 gave only one stained band with Coomassie Brilliant Blue. A major and a minor stained band were observed with the sample from the pool of fractions 62–64 and both were radioactive (Figure 2). The minor band corresponded in location to that observed with the pool of fractions 50–51. As expected, two bands corresponding to those observed in the leading and trailing edges of the radioactive peak were observed with the pool consisting of fractions 52–61. The stained bands on all three gels were radioactive and no other radioactivity could be detected on the gels.

The amino acid compositions of the biotinyl peptides present in fractions 50–51 and 62–64 are given in Table I. The peptide in pool 50–51 was found to contain ~66 residues/mol and that in pool 62–64, ~40 residues.

Dissociation of Trypsinized Transcarboxylase and Isolation of the Subunits. Since the sedimentation coefficient of the trypsinized enzyme is not altered, although a substantial portion of the $1.3S_E$ subunit has been removed, it seemed likely that the remaining portion of the $1.3S_E$ subunit might lie between the $5S_E$ and $12S_H$ subunits, thus binding them together and not being accessible to the trypsin (Figure 1). If so, the trypsinized transcarboxylase should yield the $12S_H$ subunit and the modified $6S_E$ subunits ($6S_E$(T-TC)) which could then be isolated by chromatography. In addition, the non-biotinyl peptide, designated $1.3S_E$(T-TC), and the $5S_E$ subunit might be isolated by dissociation of the $6S_E$(T-TC) subunit and by chromatography. As an aid in detecting the non-biotinyl peptides, transcarboxylase (specific activity 39) was isolated from bacteria grown in a medium containing [^{3}H]lysine and then was treated with trypsin. The trypsinization and separation of the protein from the biotinyl peptides were as described in the experiment of Figure 2. The trypsinized protein was then dissociated at pH 8 in the presence of 20% glycerol since it somewhat suppresses the dissociation of the $6S_E$ subunit to the $5S_E$ and $1.3S_E$ subunits (Ahmad et al., 1970, 1972; Green et al., 1972). The products were chromatographed on Bio-Gel A-1.5m using 0.05 *M* phosphate buffer (pH 7.0) as the eluent (Figure 3). The eluates from 1055 to 1385 ml, from 1385 to 1491 ml, and from 1491 to 1695 ml were each precipitated with ammonium sulfate (80% saturation). Sedimentation profiles of the original transcarboxylase, the trypsinized protein, the dissociated trypsinized protein, and the proteins from the three fractions of the chromatography of the dissociated protein are shown in Figure 4.

It is evident from the sedimentation velocity profiles of the original transcarboxylase (A of Figure 4) and of the isolated trypsinized transcarboxylase (B of Figure 4) that the trypsinized protein maintained a relatively intact structure although stripped of the biotinyl peptides. In this particular experiment the dissociation to $12S_H$ and $6S_E$(T-TC) subunits was not complete since some 16S material remained intact (C of Figure 4). The separation of the $6S_E$(T-TC) subunit from the $12S_H$ and 16S proteins was quite good (F of Figure 4) but the separation of $12S_H$ and 16S proteins was not accomplished (D and E of Figure 4). In other experiments in which the dissociation of trypsinized protein to $12S_H$ and $6S_E$(T-TC) subunits was essentially complete, the $12S_H$ subunit has been obtained free of the 16S component and by glycerol gradient centrifugation has been obtained free of $6S_H$ and $5S_E$ subunits. The $12S_H$ subunit obtained by these methods was found to be effective in forming active transcarboxylase in combination with $5S_E$ and $1.3S_E$ subunits (Wood et al., 1975) and was used in the experiments of Figure 6.

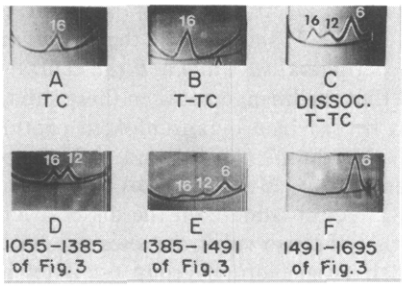

FIGURE 4: Sedimentation velocity profiles of the [^{3}H]lysine labeled transcarboxylase, the trypsinized enzyme, and the fractions from the chromatography of the dissociated trypsinized transcarboxylase (Figure 3). The sedimentations were done at 4° using ~2.5 mg of protein/ml in a 30-mm double sector cell or double sector cell with wedge window at 48,000 rpm. The pictures represent sedimentation profiles at 60–70 min. Sedimentation was from right to left. The numbers in the figures indicate the approximate sedimentation coefficients.

Dissociation of the 6S$_E$(T-TC) Subunit and Isolation of 5S$_E$ Subunit and the Non-Biotinyl Peptide (1.3S$_E$(T-TC) Subunit). The 6S$_E$(T-TC) subunit was dissociated by dialysis at 4° for 17 hr against 0.05 *M* Tris-HCl (pH 9) containing 10^{-4} *M* $PhCH_2SO_2F$. The 5S$_E$ subunit and the 1.3S$_E$(T-TC) subunit were then separated by chromatography on a Bio-Gel A-1.5m column using the same buffer. The $PhCH_2SO_2F$ was included here as well as in other procedures as a precaution against proteolysis of the peptide which might occur due to the action of contaminating proteases.

A major peak of protein and radioactivity was observed (Figure 5) followed by a broad band of low radioactivity in the subsequent fractions (note there is a tenfold difference in the two scales for cpm). Fractions 36–51 were pooled and concentrated to obtain the non-biotinyl peptide and a single band was obtained on gel electrophoresis in dodecyl sulfate (Weber and Osborn, 1969) (Figure 5). The low counts in the non-biotin peptides are in part because of the choice of [^{3}H]lysine as a label. Comparison of the amino acid composition of the 1.3S$_E$ subunit isolated earlier (Gerwin et al., 1969) as well as the preparations obtained recently[2] with that of the ~66-residue tryptic peptide (Table I) indicates that the 1.3S$_E$(T-TC) subunit may have a very low content of lysine. Likewise, it contains very small amounts of tyrosine and phenylalanine, thus, giving it negligible absorbance at 280 mμ.

The protein in fractions 12–22 gave a single peak with a sedimentation coefficient of ~6S and only a single band was observed on polyacrylamide gel electrophoresis in 8 *M* urea indicating the 5S$_E$[3] subunit was contaminated with little or no 6S$_H$ subunit. The latter subunit arises by dissociation of the 12S$_H$ subunit. Previous studies have shown that polyacrylamide gel electrophoresis in 8 *M* urea separates the constituent peptides of the 5S$_E$ and 6S$_H$ or 12S$_H$ subunits (Wood et al., 1973).

The 5S$_E$ subunit from trypsinized transcarboxylase was found to be effective in forming active transcarboxylase on combination with the 12S$_H$ and 1.3S$_E$ subunits (Wood et al., 1975).

Evidence that the Non-Biotinyl Peptide Portion of the

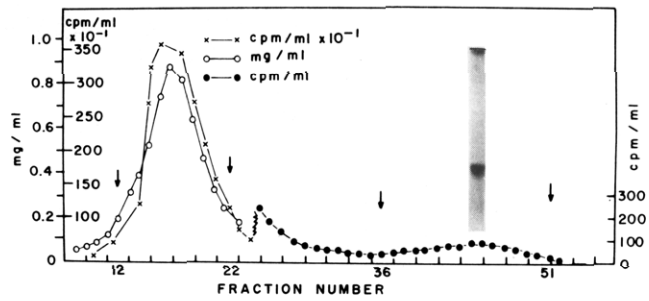

FIGURE 5: Dissociation at pH 9 of the 6S$_E$(T-TC) subunit and chromatography on Bio-Gel to obtain the 5S$_E$ subunit (fractions 12–22) and the non-biotinyl peptide (1.3S$_E$(T-TC) subunit) (fractions 36–51). The 6S$_E$(T-TC) subunit was from dissociated trypsinized transcarboxylase labeled with [^{3}H]lysine (Figure 3). The 6S$_E$(T-TC) subunit was dissociated by dialysis of 30.8 mg of protein (97,620 cpm) in 1 ml at 4° for 17 hr against 100 ml of 0.05 *M* Tris-HCl (pH 9.0) containing 10^{-4} *M* $PhCH_2SO_2F$. There was no loss of radioactivity during the dialysis. The dialyzed solution (1.3 ml) was placed on 2.2 × 120 cm column of Bio-Gel A-1.5m, 100–200 mesh. The bed volume was 425 ml and void volume ~125 ml. The column had been equilibrated with the 0.05 *M* Tris-HCl buffer (pH 9.0) containing 10^{-4} *M* $PhCH_2SO_2F$ and elution was at 4° with the same buffer. Fractions of ~3.6 ml were collected and assayed for absorbance at 280 nm and for radioactivity; 275 ml of eluate was collected prior to fraction 1. Fractions 12–22 were pooled and the protein was precipitated by addition of ammonium sulfate to 80% saturation. Fractions 36–51 were pooled and glacial acetic acid was added to obtain a concentration of 30%, the mixture was lyophilized to ~10 ml and then dialyzed against 10% acetic acid for 6 hr. It was then lyophilized to 0.5 ml and by micro biuret determination with bovine serum albumin as a standard, it contained 4.05 mg of peptide. A single band was observed on polyacrylamide gel electrophoresis in dodecyl sulfate.

1.3S$_E$ Subunit Contains the Groups which Bind the Peripheral 5S$_E$ Subunits to the Central 12S$_H$ Subunit in Transcarboxylase. It has been proposed that the 1.3S$_E$ subunit provides the links that bind together the 12S$_H$ and the 5S$_E$ subunits (Green et al., 1972; Wood et al., 1973). Evidence that this is true is presented in Figure 6. In these experiments different combinations of subunits were incubated in 0.75 *M* phosphate (pH 6.5) which promotes reconstitution from isolated subunits (Ahmad et al., 1970; Jacobson et al., 1970). The sedimentation profiles of the constituent subunits used in these studies are shown in Figure 6A–E. Each subunit gave a single peak except for the 12S$_H$ subunit (Figure 6E) from trypsinized transcarboxylase which contained some smaller subunits. The results from the reconstitution studies are given in Figure 6F–L. It is seen (Figure 6F) that the 5S$_E$ subunit in combination with the 12S$_H$ subunit did not form 16S material. However, the 6S$_E$ subunit (Figure 6G) or the 5S$_E$ and 1.3S$_E$ subunits in combination with the 12S$_H$ subunit (Figure 6H) produced the 16S form which is seen as the faster moving third peak (the 1.3S$_E$ subunit is not observed because of its small size and low concentration). These results show that the 1.3S$_E$ subunit is required for combination of the 5S$_E$ subunits with the 12S$_H$ subunit.

Since the 12S$_H$ and 5S$_E$ subunits in trypsinized transcarboxylase remain combined, it seemed likely that only the non-biotinyl peptide portion of the 1.3S$_E$ subunit is required for the linkage of the 12S$_H$ and 5S$_E$ subunits. The results with subunits from trypsinized transcarboxylase are shown in Figure 6I–L. As in the experiment of Figure 6F, when the 5S$_E$ subunit from trypsinized transcarboxylase (Figure 5) was used in combination with the 12S$_H$ subunit there was no significant formation of 16S material (Figure 6I),

[2] W. Brattin, F. Ahmad, and H. G. Wood, unpublished observations.

[3] For convenience the subunits of the 6S$_E$ subunit were designated 5S$_E$ and 1.3S$_E$. The sedimentation coefficient of the 5S$_E$ subunit has recently been determined to be ~5.8 S (Wood et al., 1975).

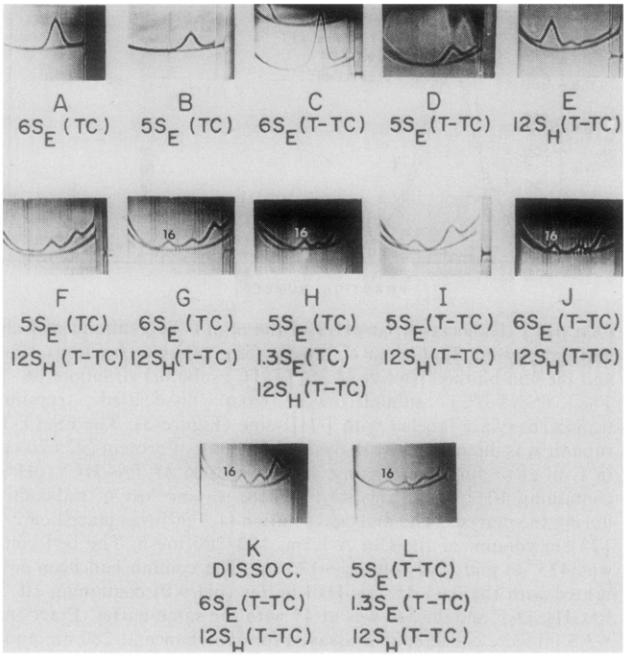

FIGURE 6: Evidence by sedimentation velocity profiles that the 1.3S_E carboxyl carrier subunit or the non-biotinyl portion is required for recombination of the 12S_H and 5S_E subunits. Sedimentation was from right to left. TC indicates subunits isolated from transcarboxylase, T-TC indicates subunits isolated from trypsinized transcarboxylase as described in the experiments of Figures 3 and 5 and Dissoc. 6S_E(T-TC) indicates the 6S_E(T-TC) had been dissociated at pH 9. Recombination of the subunits was done in 0.75 *M* phosphate (pH 6.5) at 0° for 24–40 hr usually using equal amounts (1.3 mg) of the 12S_H subunit and either the 6S_E, 6S_E(T-TC), or 5S_E subunits in ~0.5 ml of mixture. In the experiment of Figure 6H, 0.21 mg of 1.3S_E subunit was added in addition to the components of the experiment of Figure 6F. In the experiment of Figure 6D, 2.03 mg of 6S_E(T-TC) material in 0.5 ml was dialyzed for 24 hr at 4° against 2 l. of 0.05 *M* Tris-HCl (pH 9) containing 10^{-4} *M* $PhCH_2SO_2F$. To the dialyzed solution, 2.03 mg of 12S_H subunit was added in 0.11 ml followed by 0.61 ml of 1.5 *M* phosphate (pH 6.5) and the mixture was held for 40 hr at 4°. In the experiment of Figure 6L, 1.28 mg of the 12S_H subunit, 1.23 mg of the 5S_E subunit, and 0.9 mg of the non-biotinyl peptide were combined in 1.04 ml of 0.75 *M* phosphate buffer (pH 6.5) and held at 4° for 24 hr. After the incubation at 4°, the proteins were precipitated by addition of saturated ammonium sulfate to 80% saturation. After 16 hr at 0° the precipitated proteins were recovered by centrifugation and taken up with 1.1 ml of 0.1 *M* phosphate buffer (pH 6.5) (1.4 ml in experiment of Figure 6K). Sedimentation of the individual subunits (A-E) was done with ~2 mg/ml. Centrifugation was at 48,000 rpm at 4° in either a 30-mm double sector or double sector wedge cell.

but when the 6S_E(T-TC) subunit (Figure 3) was used in combination with the 12S_H subunit, 16S material was formed (Figure 6J). The experiment of Figure 6K was set up as a control to show that treatment of the 6S_E(T-TC) subunit at pH 9 does not in itself inactivate components required for the recombination of subunits. Apparently, the 5S_E subunit and non-biotinyl peptide recombined during the reconstitution forming the 6S_E(T-TC) subunit which in turn combined with the 12S_H subunit to yield the 16S material. The final evidence that the non-biotinyl peptide provides the linkage for combination of the 5S_E and 12S_H subunits was obtained using the non-biotinyl peptide isolated from the experiment of Figure 5. This peptide in combination with the 5S_E and 12S_H subunits promoted reconstitution to the 16S material (Figure 6L).

Discussion

The present results extend the evidence for the structure of transcarboxylase shown schematically in Figure 1. Previous results from electron micrographs of transcarboxylase (Green et al., 1972) showed that there is a variable gap between the peripheral subunits and the central subunit. This suggested that the linkage between these subunits is flexible and might be provided by an unfolded peptide chain. Furthermore, Northrop (1969) and Northrop and Wood (1969) had postulated on the basis of kinetic data and the inhibitory effect of oxalate on the dissociation of transcarboxylase that the two substrate sites are on different subunits and that the biotinyl group oscillates as a carboxyl carrier between the different substrate sites. The above observations in combination with the results from the electron micrographs led to the proposal (Green et al., 1972) that the biotinyl group is most likely located between the two larger subunits and that the biotin carboxyl carrier protein (1.3S_E subunit) furnishes the flexible peptide which binds the peripheral 5S_E subunit to the central 12S_H subunit.

The results presented here support this conclusion. Mild treatment of transcarboxylase by trypsin removes biotinyl peptides leaving the main structure intact with the peripheral subunits still bound to the central subunit. The product is enzymatically inactive. Polyacrylamide gel electrophoresis of the resulting biotinyl peptides gave two bands, one of which was a peptide of ~66 residues and the other of ~40 residues. It is possible that the 40-residue peptide arises by secondary cleavage of the 66-residue peptide.

The trypsinized transcarboxylase has been dissociated at pH 8 yielding the central 12S_H subunit and the peripheral subunit with a portion of the carboxyl carrier protein still attached to it [6S_E(T-TC)]. The 6S_E(T-TC) subunit has in turn been dissociated at pH 9 yielding the 5S_E subunit and a non-biotinyl peptide which has been isolated by chromatography on Bio-Gel A-1.5m. Since these are the same conditions as those used to dissociate the 6S_E subunit to the 5S_E and 1.3S_E subunits, it is assumed that the non-biotinyl peptide(s) so obtained is the residual portion of the 1.3S_E subunit.

The 5S_E subunit from either normal transcarboxylase or from trypsinized transcarboxylase does not have the capacity to bind with the 12S_H subunit from either normal or trypsinized transcarboxylase. However, addition of the 1.3S_E carboxyl carrier protein or the non-biotinyl peptide promotes the combination of the 12S_H and 5S_E subunits from either source. It is concluded that the nonbiotinyl peptide portion of the carboxyl carrier protein contains the combining domain which serves to link the peripheral 5S_E subunits to the central 12S_H subunit and that this portion is protected from tryptic action when in combination with the 5S_E and 12S_H subunits.

Chuang et al. (1975) have now firmly established that the two partial reactions of transcarboxylation occur on different subunits of transcarboxylase and that the site for the keto acids is on the metallo 5S_E subunits and the site for the CoA esters is on the central 12S_H subunit. The 1.3S_E subunit is required as the carboxyl carrier in both these partial reactions but it may be replaced by either the ~40- or ~66-residue biotinyl peptide but not by biotin or biocytin. Thus it seems likely that the exposed portion of the carboxyl carrier protein which is removed by the trypsin is the arm which permits positioning of the biotinyl group in a location adjacent to the two substrate sites on the separate subunits and permits it to serve as the carboxyl carrier between these sites and that the non-biotinyl peptide portion serves to tie the subunits into close proximity so as to facilitate this transfer.

It has been difficult to obtain a homogeneous preparation of the biotinyl carboxyl carrier protein for determination of its amino acid sequence but limited proteolysis of transcarboxylase is useful in obtaining a large segment of the biotinyl carboxyl carrier protein for determination of the primary structure. Such studies are in progress.

These studies are but one of many in which useful information has been obtained regarding the structure of enzymes by use of controlled proteolysis (Mihalyi, 1972).

References

Ahmad, F., Jacobson, B., and Wood, H. G. (1970), *J. Biol. Chem. 245,* 6486.

Ahmad, F., Lygre, D. G., Jacobson, B. E., and Wood, H. G. (1972), *J. Biol. Chem. 247,* 629.

Chuang, M., Ahmad, F., Jacobson, B., and Wood, H. G. (1975), *Biochemistry,* following paper in this issue.

Gerwin, B. I., Jacobson, B. E., and Wood, H. G. (1969), *Proc. Natl. Acad. Sci. U.S.A. 64,* 1315.

Green, N. M. (1970), *Methods Enzymol. 18A,* 418.

Green, N. M. (1972), Protein–Protein Interactions, Heidelberg, Springer-Verlag, p 183.

Green, N. M., Valentine, R. C., Wrigley, N. G., Ahmad, F., Jacobson, B., and Wood, H. G. (1972), *J. Biol. Chem. 247,* 6284.

Jacobson, B., Gerwin, B. I., Ahmad, F., Waegell, P., and Wood, H. G. (1970), *J. Biol. Chem. 245,* 6471.

Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9,* 351.

Mihalyi, E. (1972), Application of Proteolytic Enzymes to Protein Structure Studies, Cleveland, Ohio, Chemical Rubber Publishing Co.

Northrop, D. B. (1969), *J. Biol. Chem. 244,* 5808.

Northrop, D. B., and Wood, H. G. (1969), *J. Biol. Chem. 244,* 5820.

Weber, K., and Osborn, M. J. (1969), *J. Biol. Chem. 244,* 4406.

Wood, H. G. (1972), *Enzymes, 3rd Ed. 6,* 83.

Wood, H. G., Ahmad, F., Jacobson, B., Chuang, M., and Brattin, W. (1975), *J. Biol. Chem. 250,* 918.

Wood, H. G., Ahmad, F., Jacobson, B., Green, N. M., and Wrigley, N. G. (1973), in Enzyme Structure and Function, Vol. 29, Drenth, J. Oosterbaan, R. A., and Veeger, C., Ed., Amsterdam, North-Holland Publishing Co., p 201.

Wood, H. G., Jacobson, B. E., Gerwin, B. I., and Northrop, D. B. (1969), *Methods Enzymol. 13,* 215.

Evidence that the Two Partial Reactions of Transcarboxylation Are Catalyzed by Two Dissimilar Subunits of Transcarboxylase†

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ABSTRACT: The results presented here show that isolated subunits of transcarboxylase specifically catalyze the two partial reactions of transcarboxylation as shown in eq 1–3. The 12S central subunit is active in the transcarboxylation with methylmalonyl-CoA but inactive with oxalacetate and the peripheral metallo 5S subunit is active in the transcarboxylation with oxalacetate but inactive with methylmalonyl-CoA. These subunits, likewise, are specific for the reverse partial reactions; the central subunit catalyzing transfer from the carboxylated biotinyl group to propionyl-CoA to yield methylmalonyl-CoA and the peripheral subunit to pyruvate to yield oxalacetate. Thus, the central subunit contains the sites for the CoA esters (methylmalonyl-CoA and propionyl-CoA) and the peripheral metallo subunits for the keto acids (oxalacetate and pyruvate). In the overall reaction the biotinyl carboxyl carrier protein acts as a shuttle to carry the carboxyl groups between the two subunits. Biotin and certain biotin analogs are inactive in these partial reactions but the ~40- or ~66-residue biotinyl peptides, which are derived from the carboxyl carrier protein, are active. Transcarboxylase can be reconstituted from its isolated subunits and a comparison was made of the rate of the overall reaction when the subunits were assembled, as in the intact enzyme, with that obtained when the reaction was catalyzed by the nonassembled subunits. In the latter case, since the biotinyl carboxyl carrier subunit must diffuse from one subunit to the other, the overall reaction is much slower than with the assembled subunits. The reaction with trypsinized transcarboxylase from which the ~66-residue and ~40-residue biotinyl peptides have been stripped, likewise, was slow even though the biotinyl peptides were added to the reconstitution mixture. The $12S_H$ and $5S_E$ subunits remain assembled after trypsin treatment but the biotinyl peptides apparently do not combine firmly or properly with the trypsinized enzyme and the biotinyl group apparently must oscillate as a carboxyl carrier between the two sites on the subunits by diffusion.

Transcarboxylase from propionibacteria is a biotinyl enzyme, but unlike the numerous other biotinyl enzymes which catalyze fixation of CO_2 (see Alberts and Vagelos, 1972; Moss and Lane, 1971, for reviews), it catalyzes a carboxyl transfer from one compound to another without involvement of carbon dioxide. Transcarboxylase is made up of three different subunits which have been isolated and purified (Wood et al., 1975; Berger and Wood, 1975) and it is shown here that the subunits of transcarboxylase specifical-

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