The Importance of Methionine Residues for the Catalysis of the Biotin Enzyme, Transcarboxylase

ANALYSIS BY SITE-DIRECTED MUTAGENESIS*

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Almost all biotin enzymes contain the conserved tetrapeptide Ala-Met-Bct-Met (Bet, N₆-biotinyl-L-lysine). In the 1.3 S biotinyl subunit of transcarboxylase (TC), this sequence is present between positions 87 and 90. The conserved nature of these amino acids implies a critical role in the function of biotin enzymes. In order to examine the role of these conserved amino acids, point mutations in the gene encoding the 1.3 S subunit have been made by site-directed mutagenesis to generate A87G, M88L, M90L, M88T, M88C, M88A, and a double mutant A87M, M88A in the 1.3 S subunit. The mutants of the 1.3 S subunit have been reconstituted with native 5 S and 12 S subunits from Propionibacterium shermanii. The effects of mutations on the activity of TC were compared with that of TC-1.3 S wild type (WT) prepared in a similar manner. The results show that any substitution of a residue in the conserved tetrapeptide causes impairment of the rate of TC activity. Comparison of gel filtration profiles, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electron micrographs of the TC assembled with mutant 1.3 S and with wild type 1.3 S subunits showed that the impairment of the overall activity was not due to a failure of the subunits to assemble into complexes. Steady state kinetic analysis using the mutant 1.3 S subunits indicated that the kₘ values for methylmalonyl-CoA or pyruvate did not change significantly indicating that the binding of substrates is not altered. However, the kₘ values were significantly lower for mutants at positions 87 and 88 than for those at position 90. The replacement of methionine at position 88 either by hydrophobic or hydrophilic residues significantly altered the activity in the overall reaction, while similar substitution at position 90 did not dramatically alter the kₘ values. These results suggest that Ala-87 and Met-88 are catalytically critical in the conserved tetrapeptide.

Transcarboxylase (methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1)) from propionic acid bacteria (1) is a biotin and metal-containing enzyme (Mₘ = 1,200,000). The 26 S form of the enzyme consists of a hexameric central 12 S subunit (Mₘ = 360,000), six dimeric outer 5 S subunits (Mₘ = 120,000), and 12 biotinyl 1.3 S subunits (Mₘ = 12,000). The outer 5 S subunits are attached to the central 12 S subunit, three on each face, through biotinyl 1.3 S subunits (2). In addition, 18 S and 16 S forms of transcarboxylase with three and two 5 S subunits attached to the central 12 S subunit, respectively, have been reported (1). The enzyme catalyzes the following reaction which is written as the sum of two partial reactions:

\[ \text{CH}_3\text{CH}_2(\text{COO}^-)\text{COSCoA} + 1.3 \text{S} \rightleftharpoons \text{CH}_3\text{CH}_2\text{COSCoA} + 1.3 \text{S-COO}^- \] (1)

\[ \text{CH}_2\text{COO}^- + 1.3 \text{S-COO}^- \rightleftharpoons \text{OCOC}_2\text{H}_4\text{COO}^- + 1.3 \text{S} \] (2)

Sum: methylmalonyl-CoA + pyruvate \rightleftharpoons propionyl-CoA + oxalacetate (3)

The biotinyl 1.3 S subunit serves as a carboxyl carrier between the substrate binding sites on the 12 S (CoA site) and 5 S (ketoacid site) subunits. The biotin is attached to the ε-amino group of Lys-89 of the 1.3 S subunit. The biotinyl 1.3 S subunit contains a highly conserved sequence around the N₆-biotinyl-L-lysine, biocytin (Bct)³ (3, 4). The tetrapeptide, Ala-Met-Bct-Met, has been conserved in a variety of biotin enzymes throughout evolution. The only reported variations in this sequence occur in chicken and rat acetyl-CoA carboxylase where alanine is replaced by valine (5) and in urea carboxylase where the methionine on the C-terminal side of biocytin is replaced by alanine (4). The tetrapeptide was hypothesized to play a vital role either in biotinylation of Lys-89 by holocarboxylase synthetase, for the removal of biotin by biotinidase, or for the carboxylation/transcarboxylation through biotin (4). We have previously shown that substitutions at Ala-87, Met-88, or Met-90 by site-directed mutagenesis did not affect biotinylation of the 1.3 S subunit by holocarboxylase synthetase (6). Craft et al. (7) have shown that biocytin is a much better substrate for biotinidase than the biotinylated peptides, and biotinidase is not active with intact 1.3 S subunit, thus ruling out a requirement of the conserved tetrapeptide in the biotinidase reaction. In this study, we have used site-directed mutagenesis of the 1.3 S subunit to investigate the role of residues in the conserved tetrapeptide.

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³ The abbreviations used are: Bct, biocytin; TC, transcarboxylase; WT, wild type; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography.
served tetrapeptide in the assembly of TC from its isolated subunits and in the ability of TC to carry out the transfer of a carboxyl group from methylenyl-CoA to pyruvate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Methylenyl-CoA, malate dehydrogenase, reduced nicotinamide adenine dinucleotide, pyruvate, and Rose Bengal were purchased from Sigma. All other chemicals were of reagent grade.

**Preparation of Genes and Expression of Mutants of 1.3 S Biotinyl Subunits**—Our nomenclature for 1.3 S mutants is illustrated in the following example. When alanine at position 87 was replaced with glycine, it was denoted as A87G, and the TC complex, assembled from such a mutant, is called TC-L.3A S78G. The construction of 1.3 S subunit mutants 1.3 S M88T, 1.3 S M88L, and 1.3 S M90L has been described previously (6, 8). They were derived from the plasmid ptac-s which expresses the wild type gene encoding the 123 amino acid 1.3 S subunit in _E. coli_. Mutants 1.3 S A87G, 1.3 S M88C, 1.3 S M88A, and 1.3 S M87M, M88A were also derived from ptac-s using complementary oligonucleotides as substrates to substitute for the wild type (WT) sequence between an _XhoI_ site at amino acid 85 and a _BstXI_ site at amino acid 90. The complementary oligonucleotides synthesized for the _XhoI_ sites were (a) TCGAGGGATGCA and CCCTTAC for 1.3 S A87G; (b) TCGAGGCAGATCTGTT and CCGTCTGGA for 1.3 S M88A; (c) TCGAATGACTTCGCA and CTACCGG: for 1.3 S M88C; (d) TCGAAACATGCA and TGCTCAGC for 1.3 S M90C. The 5 S S/WT Subunits, the 1.3 S WT mutants, and 1.3 S WT Biotinyl Subunits—The 12 S subunit was isolated from Propionibacterium _shermanii_ as previously described (9, 10), and the recombinant WT and mutant 1.3 S subunits were isolated from _E. coli_ as described by Shenoy et al. (6). The 5 S subunit was isolated as follows. TC (150 mg with specific activity ~30.0 units/mg) was dissolved in 20 ml of 50 mM Tris-HCl buffer, pH 9.0, containing 10⁻⁷ M diethiothreitol (DTT), 10⁻⁴ M reduced glutathione, and 10⁻⁴ M phenylmethylsulfonyl fluoride (PMSF) (Buffer I) and was dialyzed against 2 liters of the same buffer. A gentle stream of nitrogen was bubbled continuously to maintain anaerobic conditions. After 18 h of dialysis, the dialysate, containing dissociated TC, was mixed with solid (NH₄)₂SO₄ at pH 9.0 to 30% saturation. The resultant precipitate was removed by centrifugation at 24,000 × g for 30 min. The supernatant, containing mostly the biotinyl 1.3 S subunit and the dimeric outer 5 S subunit, was brought to 65% saturation with (NH₄)₂SO₄. After centrifugation, the pellet was dispersed in 2 ml of 10 mM ammonium bicarbonate buffer, pH 9.0, containing 10⁻⁷ M DTT, 10⁻⁴ M reduced glutathione, and 10⁻⁴ M PMSF (Buffer II) and dialyzed against 1 liter of Buffer II for 18 h, with two changes of buffer.

The dialysate from this step was loaded onto a column packed with DE52 ion exchanger (5.5 × 10 cm) (Whatman) which had been previously equilibrated with Buffer II. The 1.3 S biotinyl subunit was eluted with 80 mM NH₄HCO₃, pH 9.0, containing 10⁻³ M DTT, 10⁻⁴ M reduced glutathione, and 10⁻⁴ M PMSF (Buffer II) and dialyzed against 1 liter of Buffer II for 18 h, with two changes of buffer.

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**Biotin Determination**—A modification of the method of Rylatt et al. (14) was used to estimate the amount of biotin in the WT or mutant 1.3 S subunits purified from _E. coli_. Biotin was quantitated by incubating 2 nmol of 1.3 S WT subunit or mutant subunit in 400 ml of 0.05 M potassium phosphate, pH 8.0, with 30 mg of pronase (Type XIV: bacterial protease, Sigma) at 37 °C for 18 h. The samples were lyophilized after terminating the reaction by boiling at 100 °C for 10 min. The lyophilized sample was dissolved in 0.2 M potassium phosphate buffer, pH 7.2, and the biotin content was estimated by the method of Rylatt et al. (14). For the estimation of biotin in the WT subunits, the sample was precipitated with trichloroacetic acid prior to digestion with pronase in order to minimize phosphate interference with the biotin determination. As all preparations of 1.3 S subunits are contaminated with the apo form, the concentration of 1.3 S used in each experiment was calculated on the basis of biotin content and not protein content. TC Assay—TC was assayed, in the forward direction as previously described (15), by detecting the formation of decarboxylase via a coupled assay system using malate dehydrogenase and measuring the decrease in NADH absorbance spectrophotometrically. The assay for the forward reaction contained a final volume of 0.3 ml: pyruvate, 2.26 mM; malate dehydrogenase, 4.5 units; NADH, 0.09 μmol; potassium phosphate, pH 6.5, 75 μmol; methylenyl-CoA, 0.9 μmol; and the assembled enzyme. Specific activities are expressed as oxalacetate formed in the forward reaction in μmol/min/mg of TC.

**High Performance Liquid Chromatography (HPLC)**—HPLC analyses were performed with a Shimadzu HPLC system equipped with a computer interface and software for the integration and analysis of the peaks in the chromatogram. An ULTROPAK TSK G4000SW (7.5 × 300 mm) gel filtration column was used for subunit separation. The elution of protein was achieved using 0.5 M potassium phosphate buffer, pH 6.5. The protein elution was monitored at 220 nm with a flow rate of 0.5 ml/min.

**Electron Microscopy**—For electron microscopy, samples were taken up in 0.1 M potassium phosphate buffer, pH 6.5, and cross-linked with glutaraldehyde (0.4%) in order to prevent dissociation during staining with uranyl acetate. The grids for electron microscopy were prepared as described by Wrigley et al. (16). All electron microscopy work described here was done at magnification of ~65,000.

**Calculation of the Number of 5 S Subunits Attached to the 12 S Subunit**—It is possible to calculate the number of 5 S subunits attached to the 12 S subunit by estimating both protein and concentrations. The protein complex of the enzyme contains one 12 S subunit, two 5 S subunits, and four 1.3 S subunits (1). From the ratio of the micrograms of protein in the TC complex to the micrograms of biotin, it is possible to calculate the number of 5 S subunits attached to the 12 S subunit. The 16 S form of TC gives a ratio of 661 which, after a calculation of 5 S subunit of the protein content of 16 S 648 μg/mmol and 4 nmol of biotin equal to 0.96 μg (4 × 0.244). Similar calculations have been made for TC complexes with varying amounts of 5 S subunits attached to the 12 S subunit. From these values, a theoretical graph has been drawn which indicates the number of 5 S subunits in various TC-1.3 S complexes based on biotin and protein content.

**Treatment of Kinetic Data**—Initial velocity was measured by varying the concentration of substrates as described by Northrop (17) in a UV 1600 Shimadzu spectrophotometer equipped with a temperature control unit. Initial velocity data from each substrate-velocity set was analyzed via a nonlinear regression software program (ENZFITTER, by Leatherbarrow, R.J. from Elseiver-BIOSOFT). The corrected data were used to construct double-reciprocal plots and were analyzed using weighted least-square regression.

**RESULTS**

**Assembly of Active Enzyme from Its Subunits**—TC assembly was achieved by incubating the 1.3 S WT subunit or a 1.3 S mutant subunit with the 12 S and 5 S subunits at 4 °C in 0.5 M potassium phosphate buffer, pH 6.5. It was necessary to maintain the phosphate concentration at 0.5 M in order to achieve assembly. The molar ratio of the 12 S:5 S:1.3 S biotinyl subunits used for the reconstitution was 1:6:12 unless otherwise stated. This is the stoichiometry of 12 S to 5 S to 1.3 S subunits in 28 S TC which contains the full complement of six 5 S subunits. In separate experiments, it was not constant that the activity of the assembled TC was the same if the proportion of 1.3 S subunits used during the assembly was increased from 12 to 18 or 24 (data not shown).

**Correlation of Time on Assembly of TC Subunits and Enzyme Activity**—The effect of variation of time on assembly and on activity using wild type and mutant 1.3 S subunits is shown in Fig. 1. As shown in Fig. 1A, the assembly is very rapid and approaches a maximum within 30 min. After 30 min, the activity of the assembled TC with either 1.3 S WT
or 1.3 S mutant subunits did not change significantly with time. There was no difference in the rate of assembly between TC-1.3 S WT and TC-1.3 S mutants. On the basis of these results, a ratio of 1:6:12 of the subunits (12 S, 5 S, and 1.3 S) and an assembly time of 24 h was used in all rate comparisons. From Fig. 1B, it is evident that there was significantly reduced activity in the overall reaction when the 1.3 S WT subunit was replaced with a mutant subunit in the assembled TC. TC-1.3 S M90L was the only TC-1.3 S mutant which displayed significant activity in the catalysis of the forward reaction.

Separation of Assembled TC Complexes from Uncombined Subunits—In the experiments reported in Fig. 1, no attempt was made to separate the TC complexes from free unassembled subunits. We next developed a technique for separating complexes from free subunits employing HPLC gel filtration chromatography. Under the conditions used (see “Experimental Procedures”), control experiments demonstrate that free 12 S, 5 S, and 1.3 S subunits were separated from the assembled TC (Fig. 2). Chromatographic separation of 12 S subunit showed that in addition to a major peak (number 3 in panel A), the preparation was contaminated with a small amount of intact TC (peak number 2 in panel A). A mixture of 5 S and 12 S subunits did not interact significantly in the absence of added 1.3 S subunits (panel C). The 1.3 S subunit eluted with the salt peak (the column bed volume is 13 ml). After reconstitution with 1.3 S WT and native 12 S and 5 S subunits, TC-1.3 S WT fractionated as a mixture of assembled forms including complexes which fractionated as if they were fully assembled 26 S forms (with six 5 S subunits attached, peak 1 of panel D) and TC with less than the full complement of 5 S subunits (peak 2) (16, 18). The elution of authentic 26 S TC is shown in panel E. Reconstitution of TC with 1.3 S M88A and native 12 S and 5 S subunits showed formation of 26 S TC and incomplete TC forms (panel F) similar to that observed with TC-1.3 S WT (panel D). None of the 1.3 S mutants used in this study affected assembly as judged by HPLC analysis. SDS-PAGE analysis and electron microscopy confirmed the composition of the material in the TC peak to contain 12 S, 5 S, and 1.3 S subunits in all cases without any unusual complexes (data not shown).

Isolation of the Complexes Formed from 12 S, 5 S, and 1.3 S Mutant Subunit and Evaluation of Their Transcarboxylase Activity—Once the conditions were established for the separation of complexes, these were isolated and activities were measured. Activities were determined for peaks 1 and 2 combined as it was not possible to completely separate the 26 S form of TC (peak 1) from the partially assembled complexes (peak 2). The results are presented in Tables I and II. In Table I, TC-1.3 S M90L and TC-1.3 S

FIG. 1. Effect of time on assembly and activity in the TC-catalyzed reaction. A, effect of time on assembly of transcarboxylase. The amount of assembly was measured using HPLC by loading a sample of a mixture of subunits on a gel filtration column at different intervals of time and calculating the area under peaks 1 and 2 as described in Fig. 2. B, effect of time on activity in the overall reaction of transcarboxylase. Assembly of each enzyme form was at 4°C in 100 μl of 0.5 M phosphate buffer, pH 6.5, containing 12 S subunit, 0.08 nmol; 5 S subunit, 0.48 nmol; and 1.3 S biotinyl subunit, 0.96 nmol, giving stoichiometric concentrations of the subunits as in 26 S TC. The transcarboxylase activity was measured as micromoles of oxalacetate formed per min⁻¹ per mg⁻¹ of TC after the indicated time of assembly.

FIG. 2. HPLC gel filtration profile of TC assembled from 12 S, 5 S, and 1.3 S subunits. The elution time for peak 1, 15.5 min (26 S TC); peak 2, 16.9 min (18 S to 24 S forms of TC); peak 3, 19.6 min (12 S); peak 4, 20.6 min (5 S); and peak 5, 23.5 min (buffer + 1.3 S). The amount of protein used for assembly and conditions for running the samples are described under "Experimental Procedures" and in Table 1.
Site-directed Mutagenesis of Transcarboxylase

TABLE I
Determination of the amount of assembled TC formed from the reconstitution mixture and efficiency of the assembled TC in catalyzing the transcarboxylase reaction

Each reconstitution mixture for assembly contained the following in nanomoles: 12 S subunit, 0.4; 5 S subunit, 2.4; and 1.3 S WT or 1.3 S mutant subunit(s) in 0.5 M phosphate buffer, pH 6.5, in a final volume of 400 μl. After 24 h, the complexed protein was separated from the 12 S, 5 S, and 1.3 S subunits by HPLC gel filtration (see "Experimental Procedures"). Units of enzyme are in micromoles of oxalacetate formed per min.

| Type of TC | Protein Activity Specific activity | mg/ml | units/ml | units/min/mg | Activity |
|------------|----------------------------------|-------|---------|-------------|----------|
| TC-1.3 S WT | 0.1 | 2.1 | 21.0 | 100.0 |
| TC-1.3 S A87M, M88A | 0.09 | 0.2 | 2.2 | 10.5 |
| TC-1.3 S M88L | 0.08 | 0.2 | 2.7 | 12.0 |
| TC-1.3 S M80L | 0.09 | 1.5 | 16.7 | 79.5 |
| TC-1.3 S M88T | 0.08 | 0.1 | 1.3 | 6.2 |
| TC-1.3 S M88C | 0.07 | 0.1 | 1.4 | 6.7 |
| TC-1.3 S M88A | 0.09 | 0.2 | 2.2 | 10.5 |
| TC-1.3 S A87G | 0.09 | 0.4 | 4.4 | 21.0 |

A87G showed around 72% and 21% of TC-1.3 S WT activity, respectively, and all other mutants showed very low activities (less than 13%) in the transcarboxylase reaction. A unit of enzyme is defined as the amount of enzyme required to produce 1 μmol of oxalacetate per min at 25 °C. The specific activities in Table I are expressed in terms of units/min/mg of protein. The values obtained were the average of five different preparations of TC complexes. The hydrophilic amino acid substitutions at residue 88 always showed lower activities than hydrophobic amino acid substitutions. For example, 1.3 S M88T had about 5% activity of TC-1.3 S WT, while TC-1.3 S M88L had 12.6% activity. The amount of protein in the separated complexes was approximately the same in all experiments (Table I) indicating that the reduced activity was not due to the failure of the mutant 1.3 S subunits to promote complex formation. To determine whether the differences in activities reflect differences in biotin content due to apo-1.3 S subunit contamination, biotin estimations of HPLC-purified TC complexes were carried out as described under "Experimental Procedures." The units of activity per nmol of biotin (Table II) correlated well with the specific activity of the enzyme (Table I) calculated on the basis of the amount of protein in the peak fractions 1 and 2 of HPLC gel filtration experiments. If a complex was formed which contained the nonbiotinyl 1.3 S apo subunit, the activity values in Tables I and II would not be similar. The activity correlation in Table I and II is independent of the forms of TC. It has been demonstrated previously that the activity per biotin is approximately the same for the two major forms of TC, namely, 18 S TC with one 12 S subunit, three 5 S subunits, and six 1.3 S biotinyl subunits (16) and 26 S TC with one 12 S subunit, six 5 S subunits, and twelve 1.3 S biotinyl subunits (18). The results indicate that the 1.3 S subunits (WT or mutants) within reassembled TC all contained biotin.

Estimation of the Number of 5 S Subunits in Transcarboxylase Formed from 12 S, 5 S, and 1.3 S WT or 1.3 S Mutant Subunits—The low activities seen with mutant TC-1.3 S subunits could have been caused by fewer than six 5 S subunits binding to the 12 S subunit. In order to determine whether the low activities were due to a reduced number of 5 S subunits on TC, the number of 5 S subunits per enzyme was determined by two methods.

1. We calculated the number assuming that peaks 1 and 2 from HPLC are made up entirely of TC and free of unbound subunits. The calculations were made as described under "Experimental Procedures." The number of 5 S subunits in the TC-1.3 S mutants and TC-1.3 S WT is presented in Table II. The results indicate that the number of 5 S subunits attached to the 12 S subunit varied from 3 to 6. This 2-fold difference may be partially responsible for the low activity with TC-1.3 S M88T mutant. Although the number of 5 S subunits varied from 3 to 6, the activities per nmol of biotin by themselves were low for TC-1.3 S mutants (Table II). For example, the TC-1.3 S mutant A87M, M88A contained 12 nmol of biotin per nmol of TC with six 5 S subunits attached to the 12 S subunit as if it were the 26 S form of TC, yet complexes containing this mutant showed only 9–11% activity per mg of protein or nmol of biotin relative to TC-1.3 S WT. In the case of TC-1.3 S M88T mutant, which contained only three 5 S subunits attached to one 12 S subunit, the activity per mg of protein or nmol of biotin did not vary drastically and was 5–6% of TC-1.3 S WT. Since the activities per biotin are approximately the same for various forms of TC, the low activities with various TC-1.3 S mutants were not due to a reduced number of 5 S subunits attached to the 12 S subunit.

2. We experimentally measured the number of 5 S subunits using SDS-polyacrylamide gel electrophoresis analysis. SDS-polyacrylamide gel electrophoresis analysis of TC, TC-1.3 S WT, and TC-1.3 S mutants showed the three bands corresponding to 12 S, 5 S, and 1.3 S before and after the isolation of the complexes. The gels were scanned and the molar ratio of the amount of 12 S, 5 S, and 1.3 S in each TC-1.3 S mutant complex was calculated and compared with that of TC-1.3 S WT (Table III). The results correlated well with the data in Table II in that the estimation of the number of 5 S subunits for every 12 S subunit was as predicted by the calculation method. The results also showed that there were no unusual

TABLE II
Determination of the amount of biotin and estimation of activity per biotin of TC and determination of the number of 5 S subunits in assembled TC

The amount of biotin was determined as described under "Experimental Procedures," and the activity was determined in the forward reaction using assembled TC as described in Table I.

| Type of TC | Biotin content Activity Activity Number of 5 S subunit |
|------------|-----------------|----------------|----------------|----------------|
| TC-1.3 S WT | 8.6 | 10.4 | 100 | 4.4 |
| TC-1.3 S A87M, M88A | 10.4 | 0.9 | 9 | 6.0 |
| TC-1.3 S M88L | 10.7 | 1.1 | 11 | 6.0 |
| TC-1.3 S M80L | 7.8 | 8.3 | 80 | 3.4 |
| TC-1.3 S M88T | 7.4 | 0.6 | 6 | 2.8 |
| TC-1.3 S M88C | 9.7 | 0.8 | 8 | 6.0 |
| TC-1.3 S M88A | 8.8 | 1.1 | 11 | 4.1 |
| TC-1.3 S A87G | 8.5 | 2.2 | 21 | 4.4 |

Table III
Determination of the number of 5 S subunits in assembled TC using densitometric scanning of sodium dodecyl sulfate-polyacrylamide gel electrophoresis

| Type of TC | Amount of subunits | Ratio 1.3 S: 5 S: 12 S |
|------------|-------------------|------------------------|
| TC-1.3 S WT | 181.8: 84.3: 21.8 | 84:1 |
| TC-1.3 S A87M, M88A | 285.0: 149.0: 23.5 | 126:1 |
| TC-1.3 S M88L | 267.0: 142.0: 22.9 | 126:1 |
| TC-1.3 S M80L | 134.0: 69.4: 21.2 | 63:1 |
| TC-1.3 S M88T | 125.0: 58.9: 21.1 | 63:1 |
| TC-1.3 S A87G | 153.0: 74.7: 19.8 | 84:1 |
complexes like 12 S-1.3 S-12 S or 5 S-1.3 S-5 S because all three subunits were present in the isolated complexes. (These complexes could have been present but not recognized during HPLC gel filtration, possibly because of similarities in molecular weight to that of partially assembled TC complexes.)

Kinetic Parameters of Wild Type and Mutant TC—The $K_m$ for methylmalonyl-CoA and pyruvate were determined as described by Northrop (17) for authentic TC from P. shermanii. The $K_m$ for methylmalonyl-CoA and pyruvate for various TC mutant complexes and for TC-1.3 S WT complex are given in Table IV. The value of $k_{cat}$ was expressed as nanomoles of oxalacetate formed per min per nmol of biotin. The results indicate that affinity of substrates was not altered in TC-1.3 S mutants, but the relative catalytic turnover rates were different from the TC-1.3 S WT. For example, the $K_m$ for methylmalonyl-CoA and pyruvate were 3.8 $\mu$M and 7.0 mM, respectively, for TC-1.3 S M88L which were not significantly different from 4.4 $\mu$M and 7.7 mM for TC-1.3 S WT. However, the $k_{cat}$ values for TC-1.3 WT and TC-1.3 S M88L were 2530 and 260, respectively, which is a 10-fold difference.

**DISCUSSION**

All biotin-containing carboxylases studied to date have a highly conserved sequence around the biocytin (biotinyl lysine). As shown in Fig. 3, the sequence Ala-Met-Bct-Met is highly conserved throughout evolution except for chicken and rat liver (not shown) acetyl-CoA carboxylase, where Ala is replaced by Val (4, 5), and urea carboxylase where Met at the C-terminal side of Bct is replaced by Ala (4). Thus, the only two completely conserved amino acids within the tetrapeptide are Met-88 and Bct-90. In this study, we considered the possibility that mutations in the 1.3 S subunit, Alac-87, Met-88, and Met-90 around Bocctin 88, could affect 5 S binding to the 12 S core and/or carboxyl transfer via biocytin of the 1.3 S subunit. The multisubunit TC can be readily dissociated and its constituent subunits can be easily separated. This property and the ability to obtain full activity after reconstitution of the isolated subunits has allowed us to manipulate the 1.3 S subunit and to examine the role of the conserved tetrapeptide in TC assembly and activity. Mutations in one of its subunits could affect function in a variety of ways, including holoenzyme assembly, substrate binding, catalytic efficiency of the overall reaction, or catalytic efficiency in either of the partial reactions. Mutations in the tetrapeptide could lead to loss of structure and hence the ability to reassemble TC and to act as a carboxyl carrier.

In order to test the competency of the mutants, a reconstitution assay was developed to determine whether mutation at Met-88 and Met-90 had any effect on the activity of TC. The results from Figs. 1 and 2 clearly show that the mutant 1.3 S subunits can promote assembly of TC. The rate of formation of assembled TC from the 1.3 S mutant subunits was comparable to that of TC-1.3 S WT showing that the relative abilities of the mutants to promote assembly has not been changed significantly. The amounts of assembled proteins produced from subunits were also similar for WT and the mutant 1.3 S subunits. As shown in Table I, although some of the mutations in 1.3 S reduced the catalytic activity, the ability to reconstitute was not affected proportionally. The results from rate of assembly, SDS-polycrylamide gel electrophoresis, HPLC gel filtration, electron microscopy, and protein and biotin content all indicated that mutant 1.3 S subunits and 1.3 S WT can assemble with 5 S and 12 S subunits equally well. These results imply that the residues around biocytin are not involved in binding of 5 S and 12 S subunits and show that the decreased activity of some mutant 1.3 S subunits was not correlated with their ability to promote assembly. We conclude that none of the mutations we have introduced into the tetrapeptide affect the rate or amount of assembled TC from individual subunits although the mutations in the tetrapeptide did affect catalytic function.

Mutations at Ala-87 and Met-88 affected the catalytic activity of TC significantly and to a greater extent than mutation at Met-90. There are, at least, three obvious requirements which must be met if a TC-1.3 S mutant is to have a rate of catalysis comparable to that in TC-1.3 S WT. (i) The 12 S and 5 S subunits must be positioned correctly in the assembled TC by the mutated 1.3 S subunit. If the 12 S and 5 S subunits dissociate because they are not bound together as firmly as in TC-1.3 S WT, the overall reaction will be slowed. (ii) Both partial reactions must be catalyzed by the

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**TABLE IV**

*Kinetic parameters of methylmalonyl-CoA and pyruvate for various TC-1.3 S WT and TC-1.3 S mutant enzymes*

| TC-1.3 S | $K_m$ for MmCoA* | $K_m$ for pyruvate | $k_{cat}$ | $k_{cat}/K_m$ |
|---------|------------------|-------------------|----------|--------------|
|         | $\mu$M | $mM$ | $min^{-1}$ | $\mu$M$^{-1}$ | $mM^{-1}$ | $min^{-1}$ |
| TC-1.3 S WT | 4.4 | 7.7 | 2530 | 575 | 329 |
| TC-1.3 S M90L | 4.2 | 7.8 | 2030 | 483 | 260 |
| TC-1.3 S M88L | 3.8 | 7.0 | 260 | 68 | 37 |
| TC-1.3 S A87G | 4.0 | 7.2 | 540 | 135 | 75 |

* MmCoA, methylmalonyl-CoA.
TC-1.3 S mutants at a rate at least as rapid as the overall reaction obtained with TC-1.3 S WT. (iii) The 1.3 S mutant subunit must be positioned in the TC complex to enable carboxyl transfer from the 1.3 S subunit to the substrate sites of the 12 S and 5 S subunits at a rate equivalent to that in TC assembled with 1.3 S WT subunits.

Amount of Biotin and Number of 5 S Subunits Present in TC-1.3 S Complexes—We tested the possibility that the number of biotins present in the assembled complexes might be less than that of TC-1.3 S WT, thus explaining the reduced activity in the overall reaction of TC-1.3 S mutants. The data from Table II indicate that similar percent activities are obtained for the TC-1.3 S mutants when specific activities are based on either protein or biotin content. Although the number of 5 S subunits attached to the 12 S subunit varied from 3 to 6, this fact did not significantly affect activity. The results clearly indicated that the complexes are formed like in TC-1.3 S WT. The differences in the overall reaction were not due to dissociation of the TC complexes as judged by HPLC gel filtration, thus eliminating requirement (i) as the source of reduced mutant activity.

Kinetic Analysis—The kinetic analysis of 1.3 S mutants indicated that the $K_m$ for substrates, methylmalonyl-CoA and pyruvate, were unchanged, while the $k_{cat}$ values decreased by as much as 10-fold. The $k_{cat}/K_m$ values indicate a decrease in the catalytic efficiency of the mutated 1.3 S subunits. Ala-87 and Met-88 seem to be more important than Met-90 in the catalytic activity of the enzyme. Met-88 looses activity when it is substituted either with a hydrophobic amino acid like Leu or Ala or a hydrophilic amino acid like Cys or Thr. A hydrophobic amino acid at position 87 seems to be important for activity because substitution of Gly at this position affected the activity considerably. Even in chicken and rat liver acetyl-CoA carboxylase, a Val, another hydrophobic amino acid, is present at this position. It should be noted that the mutation at Met-88 did not totally eliminate activity. It is possible that Met-90 might partially assume the function of Met-88 in these circumstances. It is also possible that mutation at Ala-87 or Met-88 may have altered the orientation of the biotin so that it is no longer an effective carboxyl group acceptor. Alternatively, the decarboxylation of carboxybiotin might also be affected in these mutants.

Earlier, Kondo et al. (19) showed that the methionines on either side of Bct are important for the carboxylation of biotin. They used synthetic peptides where Met was replaced by Ala to compare the rates of carboxylation of biotin. Their results indicated that the replacement of Met with Ala did not change the $K_m$ for biotin in the synthetic peptide but influenced the $V_{max}$ of the carboxylation of biotin. They also showed that the Met on either side of Bct decreased the $K_m$ for biotin in the synthetic peptide when compared to free biotin. They suggested that the cluster of sulfur atoms around biotin may have a role in carboxylation of biotin and that a subtle balance of hydrophobicity and hydrophilicity may be necessary for activity. In our studies, replacement of Met residues around Bct with other amino acids does not fully support this conclusion. The sulfur cluster around biotin may not be as important as such, since replacement of Met-90 with Leu did not change activity significantly nor did substitution of Met-88 with Cys protect against activity loss. The substitution of methionines either with hydrophilic or hydrophobic residues gave similar results suggesting that the methionine residue itself at position 88 is important. Further studies are underway to determine whether the mutations at the conserved tetrapeptide affect the carboxylation of biotin of the 1.3 S subunit using isolated subunits in partial reactions 1 and 2.

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