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Analysis of the functional domains of the 1.3 S subunit of transcarboxylase

Magner, William John, Ph.D.
Case Western Reserve University (Health Sciences), 1994

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ANALYSIS OF THE FUNCTIONAL DOMAINS OF THE 1.3 S SUBUNIT OF TRANSCARBOXYLASE

by

WILLIAM JOHN MAGNER

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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May, 1994
We hereby approve the thesis of

William John Magnier

candidate for the Ph.D.
degree.*

(signed) David Sand
(chair)

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جانيت كرونو

Joyce Gentile

J. M. Reeds

date __________________

*We also certify that written approval has been obtained for any proprietary material contained therein.
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Analysis of the Functional Domains of the 1.3 S Subunit of Transcarboxylase

Abstract

by

William John Magner

Transcarboxylase is a complex, multi-subunit enzyme found in Propionibacterium shermanii which serves as a model for the family of biotin dependent carboxylases. The enzyme is composed of three subunits termed 12 S, 5 S and 1.3 S. The genes encoding these subunits are organized in an operon. They have been cloned and sequenced (Charles G. Thornton, Ph. D. dissertation August 1987). Computational analysis demonstrated a remarkable amount of sequence conservation among family members.

This work describes E. coli expression systems for each of the genes found in this operon. The expressed recombinant proteins were studied to determine their multimeric structure and ability to catalyze the transcarboxylase partial and overall reactions. The recombinant forms of the 5 S and 12 S proteins were shown to be expressed and folded in apparently native dimeric and hexameric forms, respectively. Crude extracts of each expressed protein were shown to catalyze the appropriate transcarboxylase partial reaction.

Additional studies focused on the 1.3 S biotinyl subunit and were directed toward understanding its structural and functional domains. The 1.3 S subunit was shown to be heat stable but sensitive to mutations which significantly shortened the subunit.

Mutations were introduced into a pair of methionines at positions 88 and 90 which flank the biotinyl lysine in the 1.3 S sequence. The mutants were expressed, purified and assembled into enzyme complexes with the isolated 5 S and 12 S subunits for characterization.
Mutation of the methionine residue at position 88 was shown to affect activity in all of the transcarboxylase reactions and the halflife of the carboxybiotinyl intermediate without altering the apparent activation energy of the carboxybiotin bond. The symmetric mutations of methionine 90 resulted in moderate changes in activity and carboxybiotin halflife but significantly altered the apparent activation energy of the carboxybiotin bond. We interpret these data to indicate that the methionine residue at position 90 interacts with the biotin or carboxybiotin and may have a catalytic function while the methionine at residue 88 is more significant to the subunit structure and its interaction with the other subunits and active sites.
Dedication

For my family and friends who are so much a part of this work and my ability to complete it.
I would like to acknowledge the many people whose help, support and friendship contributed to my successful completion of this work. Thanks to: First, my family, Mom, Dad and Kevin, whose love, support and prayers carried me through all the difficulties. Jodelle, my soon-to-be wife, who listened patiently to all my frustrations and did all she could to make things easier; thanks for all those dinners in the lab, too. To all the other relatives who asked about, thought about or talked with me about the various ups and downs or graduate school. To all my friends who were available when the current frustration demanded beer! Suvinay, "George" (Pete), Harry, Phil, Brent, John,...and the soccer team. To all of my roommates (well, most of them), Umit, Michael, Greg, Harry, and Phil; the honorary and short-term roomates, Austin, Debbie, Steve, Mike, Nicky, Sue, Anna,...Derbyshire will always be remembered fondly. To my classmates, especially Austin and Lisa. To all my other friends around the country who have been so supportive over the years, especially Sonny Cino, Mike Krabak, Cheryl, Dave, MaryAnne Greco, Bill, Michael, and Debi. Everybody in the lab and the helpful, neighboring labs, Vicki, Chuck, Shou-Ih, Carol, Anne, Dan, Ganesh, Shenoy, Craig, Jo, Joan, Austin, Jesse, Xia, Jiang, Dongxiao, Pete, Yun, Lucy, Pei-Chung, PanPan, and all the others. Gerry, August and Evelyn who always helped keep us sane or at least fed. To my committee, especially David Samols, my advisor, Ganesh Kumar, Himan Sternlicht, Joyce Jentoft and Nelson Phillips as well as the earlier members, Harland Wood and Rick Miller. Other faculty members who were helpful in various ways over the years, Richard Hanson, Ganesh Kumar, Joyce Jentoft, Karen Magnus, Ken Neet, Vern Anderson. I could never have completed this work without the help of Dr. Bhami C. Shenoy and Mr. Santos Diaz.
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Transcarboxylase Introduction

Transcarboxylase (TC) was first described in 1960 by Swick and Wood as the activity responsible for carboxyl transfer in the metabolic cycle which produced propionic acid in Propionibacteria. Wood had studied the metabolism of Propionibacteria for years and had first demonstrated carbon fixation in bacteria by what is now known as the Wood-Werkman pathway (1,2). In Propionibacteria, transcarboxylase catalyzes the transfer of a carboxyl group from methylmalonyl-CoA to pyruvate forming propionyl-CoA which is transformed to propionate by CoA transferase. The "Transcarboxylase Cycle" of these bacteria takes pyruvate from glycolysis and converts it to oxalacetate which can enter the Transcarboxylase cycle and be converted to succinate and eventually to propionate or enter the Krebs cycle with a comparable result. Swick and Wood also showed that the transcarboxylase activity was ATP independent and inhibited by avidin. This led to the description of transcarboxylase as a biotin enzyme (3).

The biotin enzymes are critical to metabolism and play a central role in carboxyl transfer reactions. Biotin-dependent carboxylases have been identified in mammals, birds, plants, yeast and bacteria as playing essential roles in metabolic pathways such as gluconeogenesis, lipogenesis and amino acid metabolism. The members of the family of biotin enzymes have numerous characteristics in common (4,5). Each of the reactions catalyzed by a biotin dependent carboxylase can be expressed as the sum of two partial reactions and sequencing studies have demonstrated an extremely high level of sequence conservation among different
biotinyl enzymes. Each enzyme consists of three functional moieties - the characteristic biotinyl carboxyl carrier protein, a biotin carboxylase and a carboxyl transferase. The different members of this family carry these activities on between one and four polypeptide chains. Of the biotin-dependent carboxylases whose activities and substrates are known, most carboxyl transferases use a limited set of substrates which are either CoA esters or keto acids. In general, the biotin carboxylase activity uses HCO₃ and ATP as substrates (6). One significant difference which sets transcarboxylase apart from the other related carboxylases is that it transfers a carboxyl from one substrate to another. All other well characterized biotin dependent carboxylases fix CO₂ and require an energy source, either ATP or an ion gradient. Transcarboxylase uses a CoA ester substrate as carboxyl donor in the biotin carboxylase reaction without the need for a high energy carboxyl intermediate. The carboxybiotin is then used in the transcarboxylation reaction with the keto acid substrate.

Biotin is covalently attached to each enzyme by holocarboxylase synthetase and is always found in an ε-amino linkage to lysine in a conserved tetrapeptide which has been identified in each biotin dependent carboxylase. The striking similarities between members of this enzyme family have led to the proposal that results of studies of individual members of the family should be applicable to all members and may be useful in inferring the evolutionary relationships of the members.

The reaction catalyzed by transcarboxylase and herein referred to as the forward, overall reaction is:

\[
\text{TC} \\
\text{methylmalonyl-CoA + pyruvate} \rightleftharpoons \text{propionyl-CoA + oxalacetate}
\]
This reaction can be represented by two partial reactions as (7):

$$12 \text{ S}$$

1 - methylmalonyl-CoA + E-biotin $\rightleftharpoons$ propionyl-CoA + E-biotin-COO$^-$$

$$5 \text{ S}$$

2 - pyruvate + E-biotin-COO$^-$ $\rightleftharpoons$ oxalacetate + E-biotin

Physical characterization of transcarboxylase indicated that the holoenzyme is a complex of subunits with a total molecular weight of 1,200 kilodaltons (kDa)(3,8,9). This complex has been named the 26 S form of transcarboxylase based on its sedimentation characteristics. Three proteins were described as the components of this complex. They were also named by their sedimentation coefficients and are the 12 S, 5 S and 1.3 S subunits. A potential fourth subunit has recently been identified and, for the time-being, will be referred to as assembly promoting factor (APF)(10). APF is apparently a 60 kDa protein which was not identified previously because its molecular weight is close enough to the 12 S and 5 S monomers as to be masked on size separation media. This subunit was identified after recombinant 5 S protein was shown to be deficient in the ability to form transcarboxylase complexes in vitro. By harsher treatment and further purification, purified \textit{P. shermanii} 5 S preparations yielded an assembly activity which was subsequently termed APF. The complete characterization of APF, determination of its stoichiometry in transcarboxylase complexes and description of its role in transcarboxylase assembly are ongoing.

While authentic transcarboxylase is considered the 26 S complex, other forms of transcarboxylase have been found with sedimentation coefficients of 16 S
and 18 S. The structure of transcarboxylase as modeled from electron microscopic studies is shown in Figure 1 (11,12). This model shows a central subunit, the 12 S hexamer, with 3 subunits on each end, the 6 S subunits. It was demonstrated that the 1.3 S subunit was needed to assemble this complex (13). Electron microscopic studies by decoration with antibodies identified the biotin as being positioned between the 12 S and 5 S subunits (14). Further, it has been shown that TC complexes can be dissociated and reassembled (Figure 2) under specific conditions of ionic strength, pH, and subunit ratio (15-17). Identification of APF made it clear that this ability to assemble the dissociated subunits was also dependent on the presence of APF. Complex assembly was successful for years because the APF tightly associates with 5 S during purification and was always present as an unknown contaminant. Reconstitution with recombinant subunits requires the addition of APF. The ability to reconstitute TC complexes from isolated subunits has allowed insertion of recombinant mutant subunits into active TC complexes for further analysis.

The transcarboxylase operon was cloned and sequenced by Thornton *et al* (5,18,19) and shown to contain open reading frames for each of the three identified TC subunits under the control of a single promoter (Figure 3). The identity of the open reading frames was first identified by matching protein sequence from peptides isolated from authentic TC with the predicted amino acid sequences inferred from the DNA sequence. The gene order is 5 S, 12 S, then 1.3 S. Each gene was subsequently subcloned into plasmid expression vectors and introduced into *E. coli* hosts for expression, purification, activity and mutagenic
Figure 1. Transcarboxylase model

This model was developed by H. G. Wood et al. based on electron microscopic observations and biochemical characterization of the 26 S form of transcarboxylase. The 12 S subunit is shown in the center as a hexameric bundle. The outer 5 S dimers can be seen on both faces of the model with the 1.3 S biotinyl subunits linking the central and outer subunits.
Figure 2. Association/dissociation scheme of transcarboxylase

The equilibria which allow for transcarboxylase to be dissociated to its constituent subunits and also to be reconstituted from isolated subunits is presented schematically.
Figure 3. The transcarboxylase operon

This is a schematic representation of the operon which carries the open reading frames for the three transcarboxylase subunits as analyzed by Dr. Thornton.
studies. The identities of the genes have thus been verified by expression, immunologic reactivity and partial reaction activity. The cloned region of the transcarboxylase operon does not contain an open reading frame capable of encoding the APF. When peptide sequence data is available from APF, its gene will have to be cloned to complete the set of recombinant TC subunits.

The 12 S subunit is a hexamer in which each monomer contains two CoA ester binding sites. The molecular weight of the 12 S monomer was 60 kDa as determined by SDS-PAGE. Studies of 16 S and 18 S transcarboxylase indicate preferential loss of the outer subunits from one face before the other; this would imply that the faces of the 12 S subunit are different. Arrangement of the monomers in a parallel manner could provide the central subunit with different characteristics on each face. X-ray crystallography studies of 12 S crystals do not support this hypothesis but, as yet, are not conclusive (20).

Chemical modification and fluorescence studies indicated the involvement of tryptophan residues in substrate binding and activity (21,22). The 12 S and 1.3 S subunits interact transiently and catalyze partial reaction 1, but do not form a stable complex.

The 12 S gene was subcloned and expressed by Magner et al (23). The expressed protein was shown by SDS-PAGE and immunostaining to be the 12 S protein. Partially purified protein from this expression system was shown to support the 12 S partial reaction (reaction 1) and to be in a hexameric configuration as expressed in E. coli. Comparison of the protein sequences of the 12 S subunit and propionyl-CoA carboxylase from rat and human sources identified regions of high homology in the N terminal half of the protein but little similarity in the C terminal region (5). Since 12 S has two substrate binding sites
per monomer and the propionyl-CoA carboxylase β subunit has only one substrate binding site, it was hypothesized that one binding site would be found in the conserved N terminus and the other in the divergent C terminus. Subsequent work by Woo et al (24) has indicated that the C terminus plays a role in hexamer assembly and stability but the truncated subunits used in these studies maintained full, native CoA ester binding activity. The conserved region of the N terminal half of the protein contains several conserved tryptophans and part of a peptide which was identified by photo-affinity crosslinking as part of the active site (25). Efforts are continuing with the goal of identifying both CoA binding sites.

The 5 S subunit is a dimer in which each monomer contains one keto acid binding site and one mole of Co or Zn (26-28). SDS-PAGE indicated that the 5 S monomer had a molecular weight of 60 kDa. Fluorescence and chemical modification studies demonstrated involvement of tryptophan residues in the substrate and 1.3 S binding sites as well as the active site (21,22). Under appropriate conditions, the 5 S and 1.3 S subunits form stable 6 S complexes which catalyze partial reaction 2. The 5 S gene was subcloned, expressed in E. coli and immunologically identified with an antiserum specific for the 5 S subunit. The expressed protein has been purified and shown to be active in the 5 S partial reaction. Under the conditions of the 5 S partial reaction, the 1.3 S subunit is present in substrate amounts and interacts transiently with the 5 S for partial reaction catalysis without forming the stable 6 S complex. Comparison of the 5 S sequence with the protein sequences of oxalacetate decarboxylase and pyruvate carboxylase showed highly conserved regions which include tryptophan residues as well as cysteine and histidine residues implicated in metal binding (5). These
regions of the 5 S gene have been targeted for mutagenic analysis in studies of potential active site residues.

The 1.3 S subunit is a 123 residue monomer with a molecular weight of 12 kDa and is the biotinyl subunit (29-31). The protein sequence of this subunit was compared with the sequences of eight biotinyl proteins from 11 species. This comparison yielded striking regions of identity including a tetrapeptide surrounding the biotinyl lysine (AMKM) which appears to be characteristic of biotinyl proteins (5). This gene was cloned by Murtif et al (32) and provided the probe used by Thornton to isolate the remainder of the TC operon. Expression of the 1.3 S gene in E. coli resulted in high level expression of 1.3 S protein and biotinylation by the endogenous E. coli holoenzyme synthetase (33,34). This gene has been extensively studied by mutagenesis based on the regions of homology observed by sequence comparison. These studies were designed to elucidate the elements of 1.3 S primary structure that function in assembly of TC complexes from free subunits, recognition and appropriate biotination of the apo 1.3 S protein by holocarboxylase synthetase, activation of the biotin for carboxyl transfer, positioning of the biotin at TC's two separate active sites and movement between these sites.
CHAPTER II

The Outer and Central Subunits of Transcarboxylase

This work contributed to the following publications:


"The Effect of Deletions from the Carboxyl Terminus of the 12 S Subunit of Transcarboxylase: Catalysis of Carboxylation of the 1.3 S Subunit and Activity of Transcarboxylase," Bhami C. Shenoy, Harland G. Wood, William


Introduction

The thrust of these studies was to clone, sequence and establish expression systems for the 5 S and 12 S subunits of transcarboxylase. The work was initiated by Dr. Thornton and is summarized below. A 1.7 kb genomic fragment which had been shown to contain the 1.3 S gene was used as a probe in screening a *P. shermanii* genomic library, containing 10 kb genomic fragments in the vector pUC9, in the search for the genes of the other transcarboxylase subunits. Ultimately, a 7 kb fragment was identified which contained at least five open reading frames. The three most 5' genes mapped to the same 3.9 kb mRNA and were identified as the 5 S, 12 S and 1.3 S genes respectively. This identification was accomplished by matching of sequences predicted from the sequence of the cloned DNA with sequence known from peptide studies (35).

The 5' most open reading frame encoded the gene for the monomer of the 5 S subunit. This conclusion was reached by matching the deduced amino acid sequence from the open reading frame with sequences generated from tryptic peptides of isolated, authentic 5 S. The DNA and protein sequence are shown in
Figure 4. The 5 S subunit DNA sequence contains 1560 nucleotides encoding a protein of 519 amino acids with a calculated molecular weight of 57,793 daltons. The identification of the 5 S gene was further confirmed in 1987 when Samols subcloned an Eco RI fragment into the phagemid vector pTZ18 and demonstrated that a partially purified extract was active in the assay of transcarboxylase partial reaction 2.

The 5 S subunit was expressed from a 2.7 kb EcoRI fragment subcloned into pTZ18. This fragment encoded the entire 5 S gene and the N terminal half of the 12 S gene. This subclone, called pLac5 S, placed the 5 S gene under the control of the lac promoter. This plasmid was used to transform E. coli strain HB101 yielding clones in which the 5 S protein was detected by Western blotting. An immunoreactive band which co-migrated with authentic 5 S ($M_r$ 60 kDa) was visible in cell extracts prepared from E. coli in which the presumed 5 S was positioned downstream of the lac promoter of the vector (36). The immunoreactive band reacted specifically with anti-5 S antiserum. A crude extract of these cells was prepared and shown to be active in the transcarboxylase 5 S partial reaction. These studies have been extended to the 12 S subunit and more extensive sequence analyses toward study of active sites and other protein structure/function relationships.
Figure 4. The DNA and protein sequences of the 5 S subunit of transcarboxylase

The DNA sequence of the 5 S gene was generated by Dr. C. G. Thornton with modifications by Murtif and Magner. The protein sequence shown in standard single letter code is the sequence predicted from the DNA coding region. Portions of this sequence have been verified by matching with peptide sequence data obtained by Drs. K. G. Kumar, N. F. B. Phillips and F. C. Haase.
MATERIALS AND METHODS

Materials

DL-dithiothreitol (DTT), phenylmethyl sulfonyl fluoride (PMSF), nitroblue tetrazolium (NBT), bromochloroindolyl phosphate p-toluidine salt (BCIP), trichloroacetic acid, glutaraldehyde, pyruvate, oxalacetate, biotin, avidin, streptomycin sulfate, EDTA, p-enolpyruvate, N-ethylmorpholine, malate dehydrogenase, NADH, methylmalonyl CoA, lactate dehydrogenase and pronase (type XIV: bacterial pronase) and gel filtration chromatography standards were from Sigma Chemical Co. KH\textsuperscript{14}CO\textsubscript{3} and \textsuperscript{14}C-biotin were from Amersham Corp. IPTG, ampicillin, and restriction endonucleases were from Boehringer Mannheim. Scintillant (formula-963) was from NEN Research Products and Cytoscent from ICN. DE-53 and CM-52 were from Whatman Ltd. Biogel gel filtration chromatography resins, HPLC gel filtration column TSK SW 4000G and 3000G were from Pharmacia-LKB. C\textsubscript{g} reverse phase column was from SynChrom Inc. All other reagents were of analytical grade.

Oligonucleotides:

Oligonucleotides were synthesized by the Core Facility in the Department of Biochemistry, CWRU. The following oligonucleotides were designed for use as sequencing primers:

"5SUP" (5S upstream primer) corresponds to 5S nucleotides 1287-1303

\begin{verbatim}
CCGGCCGATCTGCTGCC
\end{verbatim}

"5SDP" (5S downstream primer) corresponds to 12S nucleotides 39-13

\begin{verbatim}
CATGGTGCTGGCGAGCTT
\end{verbatim}
"pKKH3" 23-mer pKK223-3 primer representing nucleotides 4518-41
(used for sequencing ptac1.3 mutants)
CAGGCTGAAAATCTTCTCTCATC

METHODS

Protein determination:

Protein concentrations were determined with the BioRad reagent according to manufacturer's protocol.

DNA sequencing:

DNA sequence determinations were by the Sanger dideoxy chain termination method (37) or the Maxam-Gilbert chain cleavage method (38). Dideoxy sequencing was carried out by the Klenow polymerase (37), AMV reverse transcriptase (37) or Sequenase (39) protocols.

Molecular biology:

DNA manipulations and bacterial cultures were by standard techniques (37). *E. coli* strains used were JM109, HB101, CSH26, AP1 and growth was carried out in MinA, YT or 2xYT media. For analysis of protein expression on SDS-PAGE, boiled extracts of bacterial cultures were according to procedure 52 in Gene Fusion (34).
Electrophoresis:

Denaturing polyacrylamide gel electrophoresis of DNA and proteins were carried out according to (37,40) respectively. Native gel electrophoresis of proteins (41) was carried out in 7.5% acrylamide, pH 8.9, between an upper reservoir of tris-glycine buffer, pH 8.3 and a lower reservoir of tris buffer, pH 8.3.

Western analyses:

Proteins were transferred to nitrocellulose or polyvinylidifluoride membranes by one of two methods for Western analysis. Transfer in a Hoefer electroblot apparatus required overnight incubation in four liters of tris-glycine buffer at 25 volts. The Genie apparatus accomplished protein transfers in 30 to 60 minutes, depending on the percent acrylamide and protein size of interest, at 1 amp applied current. Transferred proteins were detected immunologically by the Western procedure (34) with a goat anti-rabbit, alkaline phosphatase linked secondary antibody and color development with BCIP and NBT substrates. Total protein was visualized by staining with Coomassie Blue R250 or Amido Black dyes.

Enzyme activity assays:

Measurement of Partial Reaction 1 with the 12 S Subunit.
The partial reaction was conducted in a water bath at 25°C in 7 ml borosilicate glass vials (Fisher Scientific). The vials were placed in a tilted test tube rack so that the solution accumulated at one edge and the solutions were introduced at this edge using Hamilton syringes. The additions were made in the following order: 30 µl of 1.0 M phosphate buffer, pH 6.6, and H₂O so that the final volume will be 85 µl, then the selected amount of an aqueous solution of the 1.3 S subunit and 10 µl containing 0.6 µg of the 12 S subunit in 0.5 M phosphate buffer, pH 6.6. The reaction was started by the addition of 25 µl containing 0.0625 mM phosphate buffer, pH 6.6, 1.4 nmol of [3-14C]MMCoA and 0.03 units of MMCoA racemase (42). Only one isomer(s) of the MMCoA was enzymatically active and the racemase kept the two forms at equilibrium as the active form was utilized. The vial was immediately shaken by hand and, after 60 seconds, 50 µl of 1 M HCl was injected into the vial to stop the reaction and the vial was again shaken. After drying at 45°C under vacuum, the samples were resuspended in 200 µl of water and the radioactivities were measured by scintillation counting in 963 scintillant. In each experiment duplicate controls were included that contained the 12 S subunit but no 1.3 S subunit. The difference in acid stable counts of the control from that observed in the presence of the 1.3 S subunit was a measure of the amount of carboxyl transfer that had occurred.

Computer sequence analyses:

Analysis of DNA and protein sequences was carried out with successive generations of software. The earliest personal computer based sequence
comparisons used Pustell's program from IBI while later analyses utilized the
DNASIS, PROSIS and OLIGO programs from National Biosciences. More
sophisticated analyses were carried out with the GCG software from the Genetics
Computer Group, Madison Wisconsin. Database searches were facilitated by the
BLAST software from NCBI (43). The GenBank databases were also searched
directly via Gopher software which allowed connection to the facility in Indiana.
The BLOCKS database at the Fred Hutchison Cancer Research Center was also
searched via internet electronic mail. This database is an assembly of consensus
"blocks" characteristic of protein families.

The sequences of the transcarboxylase subunits used in these comparisons
are the predicted translation products which would result from the DNA
fragments cloned and sequenced by Dr. C. G. Thornton (18,19). The predicted
protein sequences were identified as the subunits of transcarboxylase by
comparison to peptide sequence data obtained by Drs. G. K. Kumar, F. C. Haase,
and N. F. B. Phillips. Regions of the predicted sequence which did not agree with
the sequence expected from the peptide studies or which were identified as
unreliable data by Thornton were sequenced by W. J. Magner and/or Dr. V.
Murtif Park by the methods of Maxam and Gilbert or Sanger.

Lysis and sample preparation for native analyses:

_E. coli_ JM109 containing plac12s+1.3S were grown to stationary phase,
harvested, resuspended in 10 ml/g cells of 100 mM ammonium bicarbonate buffer,
pH 7.8 with 0.01% sodium azide, 0.1 mM Na<sub>2</sub>EDTA, 2.5 mM dithiothreitol and
0.2 mM PMSF and lysed by passage through a French pressure cell. This crude
lysaté was cleared by centrifugation for 20 min at 15,000 rpm, nucleic acids and membrane components were further removed by precipitation in 5% streptomycin sulfate for 30 min. at 4°C with gentle agitation before a second centrifugation as before. The clarified lysate was then fractionated by solubility in ammonium sulfate with each step carried out at 4°C for 30 min. before centrifugation as above. The fraction which was soluble in 30% (176 g/l) ammonium sulfate was fractionated at 60% ammonium sulfate saturation (+198 g/l) with the insoluble fraction recovered after centrifugation and resuspended in 2 mls. of 250 mM acetate buffer, pH 5.5. This fraction was referred to as the 30-60% sample and was expected, from past transcarboxylase work, to contain the bulk of the 12 S protein. The pellet from the 30% saturation step was similarly resuspended and the three samples were dialyzed against 250 mM acetate buffer, pH 5.5.

Preparation of 12 S monomers:

Purified or partially purified 12 S preparations were treated in 1% SDS and 1 mM DTT overnight at 4°C to dissociate multimeric forms.

Chromatography:

Gel filtration chromatography was carried out on BioGel A0.5M and Sephadex G200 by standard methods. The columns were calibrated with molecular weight standards. A Biogel A0.5M gel filtration column (2x100 cm.) was equilibrated in 150 mM phosphate buffer, pH 6.5 and calibrated with known molecular weight proteins (blue dextran, BSA, IgG, ferritin, 12 S and 5 S).
Approximately 1 mg. of the 30-60% sample was loaded onto the calibrated column and eluted with 150 mM phosphate buffer, pH 6.5. The fractions from this elution were analyzed by western blot analysis with anti-TC antiserum and goat anti-rabbit, alkaline phosphatase conjugate as second antibody.
Results

Protein sequence analyses

The sequence of the transcarboxylase operon was the first cDNA sequence of a complete biotinyl enzyme. Sequence comparisons were constructed for the individual subunits and any corresponding sequence data available for related enzymes. These analyses have provided further description of the biotin enzyme family and identification of regions for functional study (5).

Regions of extensive sequence identity were previously described between the 5S sequence and that for oxalacetate decarboxylase (44) and pyruvate carboxylase (45,46). The newly available sequences of pyruvate carboxylase from rat (47) and human (48) allow us to extend this description of evolutionary conservation (Figure 5). Even with the addition of these sequences representing more species diversity, we still find 50-74% identity in the N terminal regions of these proteins. This conservation was anticipated given the similarity in the reactions catalyzed by these enzymes but is still remarkable in the context of the evolutionary span through which it has been maintained.

A comparable analysis was carried out for the 12S subunit sequence (Figure 6) and other biotin enzyme polypeptides containing CoA ester binding sites. We noted remarkable stretches of sequence identity when comparing the β-subunit of mammalian propionyl-CoA carboxylase (49,50) with the 12 S subunit of transcarboxylase. Significant homology between these two proteins was expected since the β-subunit of propionyl-CoA carboxylase catalyzes the reverse of the 12 S partial reaction. Transcarboxylase also
Figure 5. 5 S sequence homology

Sequence similarity comparison between the 5 S predicted protein sequence and the sequences of oxalacetate decarboxylase (44,51) and pyruvate carboxylase (46,47). The alignment is positioned for the highest degree of similarity and the numbering is based on the 5 S sequence. At several positions residues were "looped out" of the alignment to allow matching of highly similar neighboring segments, these instances are indicated in the figure. Residues common to all the sequences in the comparison are shown in bold type.
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**Legend:**
- **RS:** Propionibacterium shermanii transcarboxylase-5S subunit
- **RT:** Klebsiella pneumoniae oxaloacetate decarboxylase
- **RH:** Salmonella typhimurium oxaloacetate decarboxylase
- **RE:** Saccharomyces cerevisiae pyruvate carboxylase
- **RA:** Mju. musculus pyruvate carboxylase

---

*Three residues looped out.*

---

*Five residues looped out.*
Figure 6. The DNA and protein sequences of the 12 S subunit

The DNA sequence of the 12 S gene was generated by Dr. C. G. Thornton with modifications by Murtif and Magner. The protein sequence shown in standard single letter code is the sequence predicted from the DNA coding region. Portions of this sequence have been verified by matching with peptide sequence data obtained by Drs. K. G. Kumar, N. F. B. Phillips and F. C. Haase.
catalyzes the carboxylation of acetyl-CoA (52) which involves a partial reaction identical to that catalyzed by acetyl-CoA carboxylase. Takai et al (53) have deduced the sequence of chicken liver acetyl-CoA carboxylase from its cDNA and Lopez-Casillas et al (54) the sequence of rat liver acetyl-CoA carboxylase. These investigators have shown that there is strong homology between portions of the sequences of acetyl-CoA carboxylase and propionyl-CoA carboxylase. Similar regions of sequence identity have recently been reported between the carboxyl transferase subunit of E. coli acetyl-CoA carboxylase (55-57) and the 12 S subunit of transcarboxylase. In Figure 7, it is shown that there are homologous sequences in the acetyl-CoA carboxylase of E. coli and the propionyl-CoA carboxylase of rat with sequence 261 to 416 of the 12 S subunit and also some homology with sequence 66 to 178 of the 12 S subunit. The homology between the 12 S subunit and the β-subunit propionyl-CoA carboxylase extends over a much larger range (5,49) than it does with acetyl-CoA carboxylase. Perhaps the more confined homology with acetyl-CoA carboxylase is limited to the CoA ester site.

Expression studies

We previously reported the cloning, sequencing and initial expression of 5 S in E. coli (5,18). To improve expression, the 5 S gene was subcloned into several vectors which were transformed into different E. coli strains to determine the optimal combination for expression of the recombinant subunit. The pTZ18 vector placed the 5 S gene under the control of the lac promoter,
Figure 7. 12 S sequence homology

The protein sequence of the 12 S subunit of transcarboxylase was compared to the sequences of the β subunits of acetyl-CoA carboxylase and propionyl-CoA carboxylase. The regions of strongest similarity are presented here - the N terminal region of homology (A) and the internal region (B). The numbers in parentheses are the residue numbering of each of the sequences shown. Residues which are found in the same position in all of the sequences compared are in bold type.
Figure 7 A

rPCCβ  (88)  V E H R C A D F G M A A E K N K F P G D S V V T G R
EcACCβ (89)  K K Y K D R L A S A Q K E T G E K D A L V V H K

12S    G T I L G R P V H A A S Q D F T V M G S S A W R D A V H E G
rPCCβ  G R I N G R L V Y V F S Q D F T V F G G S L S G A H Q K I
EcACCβG T L Y G M P V V A A A F E F A F M G G S M G S V V G

12S    R R D D G T A L L T G T P F P L F F Y D S G G R I Q E G I D S
rPCCβ  C K I M D Q A I T V G A P V I G L N D S G G A R I Q E G V E S
EcACCβA R F V R A V E Q A A E D N C P L I C F S A S G G A R M Q E A L H S

12S    L S G Y G K M F F A N V K L S G V V P Q I A I I A G P C (178)
rPCCβL A G Y A D I F L R N V T A S G V I P Q I S L I M P C (203)
EcACCβL M Q M A K T S A A L A K M Q E R G L P Y I S V L T D P (201)

Figure 7 B

12S    (261)  A S F V N P N N D V
rPCCβ  (288)  A S I R E C H D P
hPCCβ  (1)   R E C H D P S D R L

hPCCβ  V P E L D T I V P L E S T K A Y N M V D I I H S V V D E

12S    G D Y L E V K A G Y A T N L V T A F A R V N G R S V G I V A
rPCCβ  R E F F E I M P N Y A K N I V I G F A R M N G R T V G I V G
hPCCβ  R E F F E I M P N Y A K N I V I G F A R M N G R T V G I V G

rPCCβ  N Q P N V A S G C L D I N S S V K G A R F V R F C D A F S I
hPCCβ  N Q P K V A S G C L D I N S S V K G A R F V R F C D A F N I

12S    P L V Q L V D V P G F L P V Q Q E Y G G I I R H G R K M L Y
rPCCβ  P L I T F V D V P G F L P G T A Q E Y G G I I R H G A K L L Y
hPCCβ  P L I T F V D V P G F L P G T A Q E Y G G I I R H G A K L L Y

12S    A Y S E A T V P K I T C L A T P T A A P T W P C A T (416)
rPCCβ  A F A E A T V P K I T V I T R K A Y G G A Y D V M S (445)
hPCCβ  A F A E A T V P K V T I T R K A Y G G A Y D V M S (155)
while the pKK223-3 vector provided the tac promoter. Expression from each
of these constructs was tested in *E. coli* strains CSH26, HB101, and AG-1.
Expression of 5 S protein was found to be vector and strain dependent.

To define conditions for maximal accumulation, growth curves of the
various vector-strain combinations were carried out at 25°C and 37°C.
Samples at varying times in culture were analyzed by western blot analysis.
Accumulation was greater at 37°C than at 25°C for all combinations of
vectors and strains and peaked at 24 hours regardless of temperature. The
clone of the 5 S gene in pTZ18 expressed in HB101 gave the highest level of
protein accumulation and is termed plac5 S (36).

The 12 S subunit was initially expressed from the expression plasmid
plac12S+1.3S which was generated when a 3.2 Kb Sph I to Bgl II fragment,
derived from the original 7.1 Kb genomic clone, was subcloned into pUC19
such that the 12 S gene was downstream of the lac promoter in the vector.
This fragment contains DNA encoding the COOH terminus of the 5 S subunit
and the entire 12 S and 1.3 S subunit genes. Total cell extracts of *E. coli*
containing this construct and a control extract derived from the *E. coli* host
strain (JM109) with the pUC19 vector were fractionated by SDS-PAGE,
transferred to PVDF membranes and incubated with an antiserum against
transcarboxylase and an alkaline phosphatase-linked second antibody. From a
comparison of the immunoreactive products with authentic subunits isolated
from *P. shermanii* transcarboxylase, it is evident that the subcloned fragment
encodes a protein which co-migrates with the monomer of the authentic 12 S
subunit and also encodes the 1.3 S subunit (Figure 8).
Figure 8. Expression and immunological identification of recombinant transcarboxylase subunits

Western analysis of a 12.5% SDS-PAGE gel with samples: 1 - plac55S/HB101 expression extract; 2 - 5 S purified standard; 3 - ptac1.3/CSH26 expression extract; 4 - plac12S+1.3S/JM109 expression extract; 5 & 6 - JM109 extract (negative control); 7 - 12 S purified standard. The mobilities of the TC subunits are indicated by arrows on the right side.
Subsequently, plac12 S+1.3 S was digested with Pst I which cleaves at position 601 in the 12 S gene and removes the 1.3 S gene. The COOH terminus of the 12 S subunit gene was reconstructed through the cloning of complementary synthetic oligonucleotides which encoded amino acids 601-604 and generated Pst I ends when annealed. The resulting subclone was termed plac12 S and was used to express the 12 S subunit alone.

Analysis of the recombinant subunits by non-denaturing electrophoresis

Each of our subunit expression systems was analyzed by native PAGE for comparison of the expressed subunits with authentic behavior as an initial verification of native folding and assembly by the recombinant proteins (Figure 9). On native polyacrylamide gel electrophoresis, authentic 5 S migrates as two diffuse bands. Our recombinant 5 S protein showed comparable behavior when an ammonium sulfate fraction of a plac5S/HB101 cell extract was subjected to native gel electrophoresis. The wild type 5 S subunits appear to behave as dimers similar to authentic P. shermanii 5 S with a native molecular weight of 120 kDa and subunit molecular weight of 60 kDa. Our expressed 12 S and authentic 12 S both migrated as indistinguishable but diffuse bands on native PAGE.

The expressed structure of 12 S was further investigated by subjecting authentic 12 S and 12 S expression extract to conditions under which the native hexamer dissociates to its monomers. The electrophoretic behavior of these monomer preparations was compared to the behavior of untreated samples and
Figure 9. Native gel electrophoresis of expressed transcarboxylase subunits

Western analysis of non-denaturing gel of expression extracts of *E. coli* carrying individual recombinant TC subunits. Lanes 1, 4 and 10 - negative control bacterial extracts; lane 2 - ptac1.3/CSH26 extract; lane 3 - plac12S/JM109; lane 5 - purified 5 S standard; lanes 6 - 9 - increasing amounts of plac5S/HB101 extract. Arrows on the right indicate the native mobilities of the individual subunits.
the behavior of a truncated form of the 12 S subunit expressed from plac12S(1-459). Immunostaining, after these samples were subjected to native gel electrophoresis, showed clear increases in mobility in the treated samples and the truncated 12 S (Figure 10). While these results could not prove that the expressed protein had formed a native hexamer in vivo, the comparison with authentic 12 S and the shift in mobility were taken as preliminary evidence in support of a native structure. The same experiments were attempted with the 5 S subunit, but the proteins degraded or precipitated and did not provide interpretable results.

Chromatographic analysis of the structure of recombinant subunits

In order for the recombinant 12 S protein to be capable of assembly into TC complexes which could catalyze the transcarboxylase partial and overall reactions, the expressed monomers must assemble to their native 12 S hexamer. Further, the 12 S hexamer must bind methylmalonyl-CoA with affinity and number of binding sites comparable to the authentic subunit and the 12 S active site must be intact and function catalytically. Beyond folding and activity of the isolated subunit, the expressed protein must be capable of productive interaction with the other subunits of transcarboxylase for formation of active TC complexes. The 1.3 S biotinyl subunit of the 6 S complex must bind to the 12 S subunit and be oriented so that it can function effectively as a carboxyl acceptor from the methylmalonyl-CoA. Even if the orientation is suitable and transfer is unaffected, the binding of 6 S subunits to both faces must be complete to have activity comparable to the fully assembled TC complex.
Figure 10. Native gel analysis of expressed 12 S subunit

Immunologic detection of 12 S proteins after non-denaturing gel electrophoresis. Samples were loaded as follows: lane 1 - purified 12 S standard; lane 2 - 12 S expression extract treated to dissociate multimeric forms; lane 3 - expression extract of plac12S(1-459); lane 4 - plac12S/JM109 expression extract.
Beyond the preliminary studies summarized above, we began examining these criteria with the first requirement that the recombinant monomers must associate to form a hexameric structure. To determine whether the expressed proteins associated to hexamers in vivo, an extract of *E. coli* JM109 containing the expression plasmid plac12S+1.3S was subjected to size fractionation by gel filtration chromatography and the native molecular weight of the recombinant 12 S was determined. A Biogel A 0.5M column (90x2cm) was chosen for its ability to clearly separate monomers (60 kDa) from hexamers (360 kDa); buffers and conditions were as described in Materials and Methods. The column was calibrated with proteins of known molecular mass, including authentic 12 S, to provide a standard curve which would correlate elution time with molecular weight. A plot of elution volume against log \( M_r \) provided a standard curve for calculation of the \( M_r \) at which recombinant 12 S eluted (Figure 11). The expression extract was run through this column and fractions analyzed by Western analysis with anti-TC antiserum (Figure 12). The resultant profile of 12 S elution was compared to the standard curve to calculate the apparent molecular mass of immunoreactive fractions. This analysis demonstrated that 12 S protein expressed in *E. coli* associates to multimeric forms, predominately the native hexamer. Some 12 S was seen in monomeric and other multimeric forms, but the predominant peak was hexameric, as in *P. shermanii*.

A second aliquot of the same 12 S extract was treated with SDS and EDTA, to dissociate multimers, and chromatographed as before. Western analyses of the two elution profiles clearly showed a shift to lower molecular weight under the influence of detergent (Figure 13). Comparison of these elution profiles with those of the molecular weight standards indicated that the detergent
Figure 11. BioGel A0.5M Calibration

The gel filtration column was calibrated with blue dextran, ferritin, immunoglobulin and bovine serum albumin. The fraction number corresponding to the peak fraction in each case is indicated by the +. The ranges of fractions containing immunoreactive 12 S proteins are indicated.
BioGel A0.5M Calibration

12 S elution range
12 S monomer elution range

\[ \text{fraction number} \]

\[ \text{in} (10^{-3}) \]

\[ N \]
Figure 12. Gel filtration chromatography of recombinant 12 S protein

Western analysis of every second fraction (23-53) from the elution of plac12S/JM109 extract from Biogel A0.5M (2x90cm). Lane 1 - 12 S purified standard; lane 2 - crude expression extract loaded on column; lanes 3 - 16 - odd-numbered fractions 23 - 53. 12 S protein eluted in fractions 33-47 with the peak at 35.
Figure 13. Gel filtration chromatography of recombinant 12 S protein after treatment to dissociate multimeric forms

Western analysis of every second fraction (23-53) from the elution of SDS/DTT-treated plac12S/JM109 extract from Biogel A0.5M (2x90cm). Lane 1 - treated expression extract loaded on column; lane 2 - 12 S purified standard; lanes 3 - 16 - odd-numbered fractions 23 - 53. 12 S protein eluted in fractions 43-51 with the peak at 47.
treated sample behaved as a single form with $M_r$ 60 kDa while the untreated sample showed multiple forms with $M_r$ 360 kDa and 60 kDa predominating.

This same process was carried out with the 459 amino acid, truncated form of 12 S. The elution profile of this shortened 12 S was consistent with that of the monomer. This result, in combination with the relative behaviors of authentic, recombinant and truncated 12 S forms on native gel electrophoresis, is supportive of the conclusion that the recombinant 12 S forms a native hexamer in vivo while the truncated form cannot assemble.

**Recombinant 12 S is active in partial reaction 1**

To determine whether expressed 12 S protein can support transcarboxylase partial reaction 1, the same crude extract used in the hexamer analysis was also tested in the 12 S partial reaction assay. In this assay, the 12 S and 1.3 S subunits transiently associate and dissociate and the 12 S subunit transfers $^{14}\text{COO}^-$ from $^{14}\text{C-MMCoA}$ to the 1.3 S biotin.

The sequence of events involved in partial reaction 1 can be represented as shown below.

\[
12\text{S} + ^{14}\text{C-MMCoA} \leftrightarrow ^{14}\text{C-MMCoA} \\
12\text{S} + 1.3\text{S} \leftrightarrow 12\text{S} \leftrightarrow 12\text{S} \\
12\text{S} + 1.3\text{S} \leftrightarrow 1.3\text{S} \leftrightarrow 1.3\text{S} - ^{14}\text{COO}^- \\
^\text{propionyl-CoA}
\]
Clearly, for the partial reaction to proceed, free 1.3 S subunits, present in substrate amounts, must associate with the 12 S subunit and the carboxylated 1.3 S subunit must dissociate from the 12 S subunit, of which only a small amount is present. Thus, factors which affect the binding capacity of the 12 S subunit for the 13 S subunit may alter the partial reaction. In addition, conformational changes of the 12 S subunit which alter its ability to transfer the carboxyl from MMCoA to the biotin of the 1.3 S subunit will affect partial reaction 1. Results with the expressed 12 S subunit indicated that it was as active as authentic 12 S from *P. shermanii*.

Thus, these studies have addressed the first three requirements for native 12 S activity -- the expressed monomers do form hexamers, these hexamers are capable of binding both 1.3 S subunits and methylmalonyl-CoA. The only requirements left to be tested are the ability to associate with the 6 S subunits to assemble full TC complexes and, quantitatively, the number and affinity of the CoA ester binding sites. Reconstituted TC complexes assembled from recombinant 12 S, wild type 1.3 S and authentic 5 S catalyzed the overall reaction as efficiently as authentic TC thus demonstrating the ability of the recombinant protein both to associate with 6 S complexes and to catalyze the complete transcarboxylase reaction.
DISCUSSION

The cloning of the transcarboxylase operon provided the genes for all three subunits and basic information about their context and expression in Propionibacterium shermanii. Analysis of this sequence data in the context of comparable, evolving data for other members of the biotin enzyme family has allowed description of regions which have been evolutionarily conserved among enzymes with similar activities and substrates. While this homology is interesting in its own right, it has provided a basis for hypotheses, experimental design and rational targeting of mutagenesis in attempts to characterize substrate binding and active sites in the absence of structural data. To take full advantage of this opportunity, the genes must be expressed to provide a source of the cloned protein and the expressed protein must have characteristics comparable to the authentic, native protein.

Cloning and expression of the 1.3 S protein were previously reported, but this has been repeated with the 1.3 S gene as part of the transcarboxylase operon. Studies of this gene continue in order to characterize regions of interaction with the other subunits of TC, and with holocarboxylase synthetase as well as regions involved in biotinyl carboxyl carrier activity.

Characterization of the expressed product of the previously subcloned 5 S gene now provides a system in which recombinant protein is expressed in an active, apparently native form which will be useful in the characterization of keto acid binding sites, dimer interfaces and regions of interaction with the other subunits of transcarboxylase.
A similar expression system was constructed for the 12S subunit of transcarboxylase. The recombinant 12 S protein has now been expressed alone and in combination with the 1.3 S subunit. Characterization of the expressed 12 S protein has demonstrated that this system provides an apparently native 12 S subunit which forms a hexamer in vivo and can be isolated in active form. Woo et al (24) addressed the final questions of the characterization of the recombinant 12 S and showed that the number of CoA ester binding sites and their $K_M$ values are indistinguishable from authentic 12 S. The availability of this 12 S expression system now provides an opportunity for exploration of the putative CoA ester binding sites and regions of interaction necessary in hexamer formation and interaction with the other subunits of TC by selected mutations of the 12 S subunit gene. Targets for these studies have been defined by a combination of data from the sequence homology analyses presented above, preliminary characterizations of the two recombinant subunits and extensive chemical modification studies.
CHAPTER III

The 1.3 S Subunit, C Terminal Studies and Purification

This work contributed to the following publications:


"Mutagenesis Affecting the Carboxyl Terminus of the Biotinyl Subunit of Transcarboxylase: Effects on Biotinylation.", Vicki L. Murtif and David Samols (1987) J Biol Chem 262, 11813-6 (mutant ptacl.3(1-123AP))

INTRODUCTION

This chapter will deal with a variety of topics related to the 1.3 S subunit. My contributions to our understanding of 1.3 S-avidin chromatography, heat stability of the 1.3 S subunit, the 1.3 S "hinge" region and the 1.3 S COOH-terminus will be described. These investigations will show that (i) both
biotinylated and non-biotinylated (apo) forms of 1.3 S subunit can specifically bind to avidin (monomeric)-agarose columns thus demonstrating the ability of a non-biotinylated protein to bind avidin and (ii) separation of apo and biotinylated 1.3 S was achieved by hydrophobic interaction chromatography.

The gene for the 123 residue 1.3 S biotinyl subunit has been cloned, sequenced, and expressed in E. coli in active form (32). In the biotinyl 1.3 S subunit, the biotin is attached in an amide linkage to the ε - amino group of Lys 89, a post-translational reaction catalyzed by holocarboxylase synthetase (33,34). The synthetases from various sources have broad specificity. For example, the synthetase from P. shermanii catalyzes the biotinylation not only of apo TC but also apo propionyl-CoA carboxylase from rat liver (50) and apo acetyl-CoA carboxylase from yeast (58). Likewise, the holocarboxylase synthetase from E. coli showed a broad specificity (33). Acetyl-CoA carboxylase is the only biotinyl enzyme found in E. coli, nevertheless, when the 1.3 S gene was expressed in E. coli, 1.3 S was correctly biotinylated by the endogenous synthetase (32,34). Overexpression of the 1.3 S subunit in E. coli yields a mixture of apo and biotinylated forms.

Avidin affinity chromatography

Avidin-biotin technology is widely applied in several areas of research. The underlying principle of these procedures stems from the fact that the equilibrium dissociation-constant of the biotin-avidin complex is exceptionally low, on the order of 10^{-15} M. This tight binding occurs through a highly stable, non-covalent interaction (59,60). Avidin-biotin systems have been used for
affinity cytochemistry, histochemistry, pathological probes, diagnostics, immunoassays, hybridoma technology, blotting technology, bioaffinity sensors, gene probes, cross linking agents, affinity targeting, affinity perturbation, drug delivery, fusogenic agents, immobilizing agents, selective retrieval, selective elimination, flow cytometry, and affinity chromatography [see reviews by Wilchek and Bayer (61-63)]. Bioseparation of naturally occurring biotinyl proteins and chemically biotinylated proteins on immobilized avidin columns takes advantage of the preferential binding of the biotin moiety to the columns and its subsequent release by addition of free biotin. It is generally assumed that the interaction of non-biotinyl proteins is either weak or absent. This approach is used routinely to purify biotin enzymes, such as pyruvate carboxylase (64), propionyl-CoA carboxylase (65), transcarboxylase (TC) (66) and acetyl-CoA carboxylase (56). It has also been used to purify subunits of transcarboxylase (66) and of acetyl-CoA carboxylase (56) through binding to avidin columns followed by dissociation of the subunits.

Further studies described here were designed to characterize the structural and functional properties of the 1.3 S subunit of transcarboxylase and of specific regions of the 1.3 S subunit in its interaction with holocarboxylase synthetase, other transcarboxylase subunits and avidin.

**Heat treatment of the 1.3 S protein**

The 1.3 S subunit is a relatively small protein whose secondary structure has not yet been determined. Purification procedures have been adapted over years of studying this protein and, currently, it is subjected to 0.1% trifluoroacetic
acid and acetonitrile during reverse phase HPLC purification. The protein recovered after this procedure is fully active and not different from that purified by milder conditions. The effect of heat on 1.3 S was never studied but, in light of these facts, we expected the protein to be highly stable to heat. Wild type 1.3 S was used for studies of the effect of heat on crude extracts and the effect of heat on the ability of 1.3 S proteins to reconstitute TC complexes and catalyze the overall reaction.

The 1.3 S "hinge" region

Specific regions of the 1.3 S subunit were targeted for analysis of functional significance based on sequence homology studies and analysis of peptides in activity assays.

A hinge region has frequently been discussed as a mechanism whereby the 1.3 S subunit could provide the flexibility for biotin to move between the two active sites on the central and outer transcarboxylase subunits. Comparison of biotinyl protein sequences indicated significant conservation of repeating glycine and valine residues as well as a proline-alanine-proline triplet between residues 58 and 84. The structure of proline is known to cause severe constraint on the flexibility of polypeptide chains at the point at which it is incorporated. The discovery of this conserved triplet led to consideration of this as the site of a bend which could function in positioning the biotinyl moiety (5). Beyond the bend for positioning, the conservation of regularly spaced glycine and valine residues could provide a flexible structure which could allow for movement between active sites on the other subunits. Several of these glycine/valine repeats were also found in
analogous positions in the E2 lipoyl subunits of various dehydrogenases and H proteins (67). These lipoyl domains have been shown to fold so that the lipoic acid is positioned on a flexible arm similar to what we have proposed for the 1.3 S protein (5,68,69).

Shenoy et al (70) produced a series of biotinyl peptides from the 1.3 S subunit and studied their capacity to support the transcarboxylase partial reaction activities. It was shown that the peptide containing residues 59-101 was fully active while the peptide containing residues 78-101 was inactive. The conclusion from these studies was that a feature found between residues 59 and 78 is necessary for enzyme function. This region contains the second proline of the conserved triplet as well as two of the gly-val repeats.

Since these studies involved peptides, the amino terminal segments required for complex assembly were absent and only partial reaction activity could be assayed. Presumably the transient association of the subunits in these assays, in which the 1.3 S subunit or peptide acts as a second substrate, is independent of the biotin movement required in transcarboxylase activity. To analyze the contribution of this region, and specific residues in it, to biotin motion, or any other aspect of transcarboxylase activity, this region was subjected to mutagenic analyses. These analyses allow specific alterations or deletions while maintaining the context of the protein to allow for transcarboxylase complex assembly and assay of all component activities as well as overall reaction activity.

\v

The 1.3 S carboxyl terminus
Comparison of the biotin carboxyl carrier protein sequences from the various members of the biotin enzyme family revealed an intriguing similarity in the number of amino acid residues from the biocytin to the carboxyl terminus (34). The carboxyl terminal residue, however, did not demonstrate significant homology. This apparent conservation of length or spacing led us to consider that this region could be a protein folding domain which would function in recognition by holocarboxylase synthetase or in direction of the synthetase to lysine 89.

It had been shown that the N terminal region of 1.3 S was involved in assembly of the subunits to form the 26 S transcarboxylase complex (30). There were indications that the C terminus might also play a role in complex formation and stabilization. To investigate what minimal sequence of 1.3 S was required for proper biotinylatation and what assembly or activity N terminal truncated 1.3 S subunits could catalyze, Murtif made ptac1.3t constructs which would express 1.3 S subunits with N terminal truncations. These shortened 1.3 S proteins were studied for their ability to be biotinylated and to catalyze the transcarboxylase partial reactions. When the construct designed to express only residues 85-123 was expressed in E. coli, no immunoreactive protein or in vivo biotination could be observed. While we were interested in what characteristics of the segment 55-85 prevented a protein lacking this segment from accumulating, we still wanted to remove all residues N terminal of the conserved tetrapeptide and determine function in the partial reactions and in association with the other subunits. Since expression of the truncated construct was unsuccessful, a fusion protein was designed to allow production of the 1.3 S 85-123 peptide.

The expression vector pJG200, designed by Deepak Bastia (71,72) to provide a fusion protein expression vector with a cleavable linker, was chosen for
these studies. The fusion protein produced by this vector would have the inserted protein as its N terminus, a collagen linker and the bulk of the β-galactosidase protein. A fusion protein produced in this way could be purified by affinity chromatography on an anti-β-galactosidase column and cleaved by collagenase treatment to yield the protein of interest and a major β-galactosidase fragment. This system was chosen as an alternative to expression of a truncated construct of the gene after bacteria containing the shortened gene failed to accumulate the desired protein. Fusion protein expression systems fuse the coding sequence of the peptide of interest to a much larger portion of a freely soluble protein to mask a peptide which would otherwise be a target for proteases, poison cells expressing it or aggregate. We chose this particular vector because our peptide would be linked to the fusion protein by a cleavable peptide thus allowing purification of the peptide of interest free of the fusion partners.
MATERIALS AND METHODS

Materials

Restriction enzymes and nucleotides were from Boehringer Mannheim Biochemicals and provided by the CWRU Dept. of Biochemistry stock room. Sequenase vers. 1.0 and 2.0 was from United States Biochemicals, Cleveland, Ohio. Pronase, non-specific protease, and activity assay substrates were from Sigma. [14C]Biotin was from Amersham. The Progel TSK Ether 5PW column was from Supelco and the C4 reverse-phase column was from Synchro. Other materials were of analytical grade.

Methods

General molecular biology methods

Basic molecular biology procedures, such as restriction digestion, kinase, phosphatase and ligation reactions were carried out as described in Molecular Cloning: A Laboratory Manual (37). Ligations in agarose were developed by various laboratories during the timespan of this work. Several different protocols were used as the method progressed. The final and most successful version published in 1991 by D. V. R. Kalvakolanu and W. H. Livingston III (73). Transformation of E. coli DNA constructs was variously accomplished by the Hanahan method (74), the CaCl method (37) or electroporation (BioRad). DNA
preparations were accomplished according to Maniatis (37) or Qiagen® Phage growth was according to standard procedures (75, 76). Phagemid growth was similar to phage growth except that the bacteria used were plac1.3/JM109 and cultures were infected with M13 helper phage of strain M13K07. Phage and phagemid DNA were isolated and purified by standard procedures (75, 76). Electrophoresis of DNA on agarose and acrylamide gels, native and denatured, were performed according to standard procedures (37). SDS-PAGE of expression extracts and purified proteins were according to Laemmli (40) in mini-gel (BioRad) or larger formats. DNA fragments were recovered from agarose gels by binding to and elution from glass beads (37) or electroelution (IBI).

Oligonucleotide preparation

Oligonucleotides were synthesized by P. L. deHaseth, Dept. of Biochemistry, CWRU or the CWRU Biochemistry Core Facility. Crude products of oligonucleotide synthesis were separated on a 20% acrylamide, 7M urea polyacrylamide gel. Full-length oligonucleotides were visualized by ultraviolet shadowing followed by recovery from the chosen gel slice by elution with 0.5 M ammonium acetate, 1 mM EDTA and purification on a small (330 mg.) C18 column. More recently, the oligonucleotides were purified by the Core Facility prior to delivery. Individual oligonucleotides and their use are described below.

Mutagenesis
Mutagenesis was accomplished by one of two basic procedures — cassette mutagenesis or primer-directed, site-specific mutagenesis. For cassette mutagenesis, equimolar amounts of purified complementary oligonucleotides were mixed together in TE buffer, heated to 65°C and allowed to slow cool to form a double-stranded DNA cassette which could be ligated into restriction endonuclease digested plasmid by standard subcloning procedures. When mutagenesis was primer-directed, the mutagenic oligonucleotide was phosphorylated with T4 kinase by the standard reaction, mixed with uracil-containing ssDNA, heated to 65°C and allowed to anneal by slowly cooling to room temperature. After annealing, synthesis buffer was added for second strand synthesis catalyzed by T4 DNA polymerase (77,78) or Sequenase® (39). Use of the uracil-containing template strand provided a selective advantage to the newly synthesized strand after transformation into wild type E. coli (75,76).

Construction of ptacl1.3(1-123AP)

In the construction of the 1.3 S-ß-gal fusion construct in pJG200, the coding region to be inserted must be carried by a DNA fragment with BamH1 ends. The following oligonucleotide sequences were used for construction of the 1.3 S C terminal coding region with an in-frame BamH1 restriction endonuclease recognition site at the stop codon:

MBM-N:
\[
CGCGGTGCAGGCGGTCAGGGTCTCATCAAGATCGGGGATCCATAA
\]
MBM-C:
\[
CGCGTTATGGATCCCCGATCTTGATGAGACCCTGACCGCCCTGCAC
\]
When annealed, this pair of oligonucleotides forms a double-stranded cassette with Mlu1 ends, the desired BamH1 site and the wild type coding sequence of amino acids 112-123. This synthetic cassette was inserted into Mlu 1 digested ptc1.3 (1-112) and ligated to form ptc1.3 (1-123AP). Putative clones were screened to identify the new mutant by colony hybridization with $^{32}$P end-labelled oligos MBM-C and MBM-N.

Construction of ptc1.3(85-123AP)

The ptc1.3(1-123AP) construct encoded the entire 1.3 S protein plus two extra residues which resulted from the reading frame which included the BamH1 site before the stop codon. To produce a construct capable of encoding a fusion protein with the 1.3 S 85-123 segment, an appropriate start codon had to be inserted 5' of the codon for amino acid 85. The oligonucleotides used to construct the N terminus of the 85-123 construct were: X-N: AATTCATGC and X-C: TCGAGCATG. These complementary oligonucleotides anneal to form a double-stranded DNA cassette with an EcoR1 5' overhang, an ATG start codon, and an Xho1 3' overhang. When ligated to EcoR1 and Xho1 digested ptc1.3 DNA, this synthetic cassette will provide the start codon for expression of a 1.3 S peptide N terminally truncated at residue 85. Insertion of this synthetic oligonucleotide cassette was carried out as described above and putative clones were identified by screening small plasmid DNA samples by restriction endonuclease digestion.

Construction of "hinge" region mutants
Oligonucleotides synthesized for mutagenesis of the hinge region are shown below. These mutagenesis experiments used primer-directed mutagenesis of single stranded phage or phagemid DNA templates. Synthesis of the second strand was visualized by agarose gel electrophoresis since the DNA mobility decreased as synthesis progressed. The protocols used were as described above with either placl.3S phagemid DNA or M13-1.3S phage DNA as template. Mutagenic primers are listed below with the designation of native residue, resultant residue and diagnostic restriction endonuclease site incorporated in the mutant design.

Mutations of both of the proline residues of the P-A-P (58-60) sequence of the conserved hinge region:

- P→W + BglII  
  CCGGCCAGCCAAAGCCAGATCTCGCCC
- P→A + PvuII  
  CCGGCCAGCGCAGCTGCAATCTCGCCC

Mutation to delete segment encoding residues 58-64:

Δ58-64:  
GATCTTGAGACGGTAATCTCGCCTCTCC

In this case, the mutagenic primer would lack the segment of DNA to be deleted but would anneal perfectly on both sides of the segment to be deleted. When a mutagenic oligonucleotide of this type was annealled to its template, the segment to be deleted would loop out from the double-stranded segment and the newly synthesized strand would lack the entire loop.

Western blotting
Protein expression was analyzed by Western blot analysis (34). Antisera used in the detection of immunoreactive proteins were anti-TC (79) or anti-β-gal from CalBiochem. β-gal activity was assayed by the procedure of Yarborough et al (80). Biotin incorporation in expressed proteins was analyzed in 14C-biotin-containing cultures (32).

*In vivo* biotination

Expression cultures were grown overnight in minimal media supplemented with 0.4 μM 14C-biotin, harvested and lysed by boiling in Laemmli sample buffer. The culture volume harvested from each culture was standardized by absorbance at 600 nm. These lysates were then fractionated by 15% SDS-PAGE. After electrophoresis, the gels were incubated in 1M salicylate with 1% glycerol, dried onto paper and autoradiographed with Kodak XAR5 xray film. An alternative method has been developed which replaces the fluorography, 14C-biotin and minimal media. In this method, cell lysates were prepared from standardized volumes of cultures, electrophoresed and visualized by Western blotting. For this purpose, a goat antibody to biotin was purchased from Sigma and used as the first antibody. The second antibody was a rabbit anti-goat antibody conjugated with alkaline phosphatase. The bound antibody was visualized with BCIP and NBT from USB Biochemicals.

Protein purification methods
Cells were grown in 2 X YT medium (37) containing one mg biotin per liter. Cells were harvested at stationary phase and suspended (wet weight/volume ratio 1:5) in 0.1 M ammonium bicarbonate buffer, pH 8.3, containing 1 mM EDTA, 2 mM PMSF, and 0.01% NaN₃ (buffer A) and were lysed by passage through a French Press. The extract was centrifuged at 27,000 x g for 15 min. at 4°C. The purification procedure was carried out at room temperature unless otherwise stated. The clear supernatant was treated with an equal volume of 10% streptomycin sulfate at 4°C with stirring for 20 min. The precipitate was removed by centrifugation at 27,000 x g for 15 min. and the supernatant was fractionated using ammonium sulfate. The protein pellet resulting from 30-60% ammonium sulfate saturation contained most of the 1.3 S subunit. This fraction was dissolved in buffer A and dialyzed against 100 mM potassium phosphate buffer, pH 6.8, containing 0.15 M NaCl (buffer B) for 24 h with three changes at 4°C. The dialyzed sample was loaded on regenerated avidin (monomeric)-agarose column (bed volume 35 ml) which was previously equilibrated with buffer B. Regeneration of avidin (monomeric)-agarose columns was carried out as follows: The column was washed with 5 bed volumes of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.15 M NaCl and 1 mg/ml biotin to block all non-exchangeable biotin sites, followed by 10 column volumes of 0.1 M glycine-HCl buffer, pH 2.0, to remove biotin from the exchangeable biotin binding sites. The column was then equilibrated with buffer B before loading the sample. After loading, the column was washed with buffer B until the absorbance of the effluent was less than 0.05 at 220 nm. The elution of the bound 1.3 S subunit was then achieved either using 0.1 M phosphate buffer, pH 6.8, containing 0.15 M NaCl and 1 mg/ml biotin (biotin-containing buffer) or 0.1 M glycine-HCl buffer, pH 2.0
(glycine-HCl buffer). Typically from 10 g of cells about 4.6 mmol of 1.3 S subunit was obtained. Avidin (monomeric)-agarose binds 10-15 nmol of 1.3 S subunit per ml of the gel.

HPLC analysis was performed with a Shimadzu HPLC system equipped with a computer interface and software for the integration and analysis of the peaks. An ULTROPAC TSK SW4000G gel filtration column was used for separation of the reconstituted TC from free 12 S and 6 S subunits and also for analysis of the heat-shocked 12 S proteins. The elution of the protein was achieved by using 0.4 M potassium phosphate buffer, pH 6.5 containing 0.1 mM EDTA and 0.1 mM DTT for reconstituted TC and 10 mM phosphate buffer, pH 6.0, containing 5% glycerol, 0.2 mM EDTA and 0.1 mM DTT for heat-treated samples with a flow rate of 0.5 ml/min. The protein elution was monitored at 220 nm.

Purification of 1.3 S and its mutants was routinely performed on a SynChrom C4 reverse phase 250x4.6 mm HPLC column with a 50x4.6 mm guard column. Purifications were run on the Dupont 850 HPLC system with buffer A as 0.1% TFA in water and buffer B as 0.1% TFA in acetonitrile at a flow rate of 0.9 ml/min. Elution of 1.3 S proteins was accomplished with a program in which a gradient of 0-40% buffer B was allowed 10 minutes followed by a 60 min. elution at 40% B, a rapid gradient to 100% B over 5 minutes, a five minute hold at 100% B and a five minute gradient to return to 0% B. Absorbance at 220 nm. was monitored with a Shimadzu SPD-6A UV detector. 1.3 S proteins split into two peaks, the first, centered at 49 min. in segment 2, was predominately apo while the second, centered at 51 min. in segment 2, was predominately biotinyl. As discussed in Shenoy et al (81), this separation was not complete but did provide
nearly pure 1.3 S protein whose biotin content could be assayed. Complete separation of the two 1.3 S species could only be achieved by hydrophobic interaction chromatography (81).

Assay of biotin content

A modification of the method of Rylatt et al (82) was used to estimate the amount of biotin in the TC samples. For the estimation of biotin in the TC samples, the protein was precipitated with trichloroacetic acid prior to digestion with pronase in order to minimize interference by the high phosphate content of the TC reconstitution assays. Biotin was quantitated by incubating TC (equivalent to 2 nmol of 1.3 S subunit) in 400 μl of 0.2M N-ethylmorpholine-acetate buffer, pH 8.0 with 50 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 samples were dissolved in 0.2 M potassium phosphate buffer, pH 7.2 and the biotin content estimated by the method of Rylatt et al (82) in which avidin is used in a scheme comparable to standard radioimmunoassays.

Protein concentrations were determined by the Rose Bengal Protein assay (83).
RESULTS

Purification of 1.3 S (both apo and biotinylated) subunits

The recombinant 1.3 S subunit was purified from *E. coli* cultures containing the 1.3 S subunit expression plasmid ptac 1.3t.

The 1.3 S subunit isolated from the avidin-monomeric agarose column displayed a single band on SDS PAGE with a molecular weight of 12,000 (Figure 14). Western Blot analysis using a polyclonal antiserum raised against *P. shermanii* 1.3 S subunit (79) showed that the purified recombinant protein was immunologically related to the authentic 1.3 S subunit (Fig. 15, lane 2). The elution of avidin (monomeric)-agarose columns with the biotin-containing buffer eluted almost all the proteins which bound to the column. Subsequent washes with the glycine-HCl buffer did not contain any 1.3 S protein as determined by Coomassie blue staining of SDS PAGE gels but low levels were detected by Western Blot analysis (Fig. 15, lane 3).

Biotin concentration was estimated by a modification of the Rylatt method (82). For the estimation, the 1.3 S purified as described above was desalted on a Bio-Gel P 6DG column, followed by thorough dialysis against water with three changes at 4°C and lyophilization. The samples were then taken up in 0.1 M N-ethylmorpholine acetate buffer, pH 8.0, and subjected to pronase treatment prior to biotin determination. The results are shown in Table I and indicate that the purified protein from avidin (monomeric)-agarose columns was only 28% biotinylated.
Figure 14. SDS-PAGE of 1.3 S subunit purified by avidin (monomeric)-agarose affinity chromatography

Coomassie stained 15% SDS-PAGE: lane 1 - molecular weight standards (myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 97,400; bovine serum albumin, 66,300; glutamate dehydrogenase, 55,400; lactate dehydrogenase, 36,500; carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; lysozyme, 14,400); lane 2 - crude extract; lane 3 - 1.3 S eluted with biotin-containing buffer; lane 4 - 1.3 S eluted with glycine-HCl buffer; lane 5 - authentic 1.3 S subunit.
Figure 15. Western analysis of SDS-PAGE of 1.3 S subunit purified by avidin (monomeric)-agarose affinity chromatography

Western analysis of 15% SDS-PAGE: lane 1 - crude extract; lane 2 - 1.3 S eluted with biotin-containing buffer; lane 3 - 1.3 S eluted with glycine-HCl buffer; lane 4 - authentic 1.3 S subunit.
### Table I

Biotin content of 1.3 S protein purified by avidin (monomeric)-agarose affinity chromatography or by hydrophobic interaction chromatography (HIC)

<table>
<thead>
<tr>
<th>1.3 S</th>
<th>Biotin content (nmol biotin/nmol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avidin purified</td>
<td>0.28</td>
</tr>
<tr>
<td>HIC peak 1</td>
<td>0.0</td>
</tr>
<tr>
<td>HIC peak 2</td>
<td>1.02</td>
</tr>
</tbody>
</table>
The above results suggest that the avidin-monomeric agarose column was not specific for biotinyl 1.3 S since the non-biotinylated 1.3 S subunit was also bound. As a consequence, methods were developed to separate the apo from the biotinylated form of the 1.3 S subunit as follows.

To test the ability of reverse phase HPLC to separate apo and biotinylated 1.3 S subunits, the sample purified on the avidin (monomeric)-agarose column was dissolved in water and loaded on a C4 reverse phase column. The elution of the 1.3 S subunit was achieved by using a linear gradient of 0.1% TFA in water and 0.1% TFA in acetonitrile as described under Methods. The elution profile is shown in Figure 16. The profile indicates two distinct major peaks with some minor contaminating peaks. Measurement of the biotin content of peaks 1 and 2 demonstrated that peak 1 contains 0.1 nmol of biotin per nmole of protein and peak 2 contains 0.7 nmol of biotin per nmole of protein, thus indicating that peak 1 contains apo and peak 2 biotinylated 1.3 S protein and that these peaks are incompletely separated. Since it was not possible to further resolve the two peaks, an alternate separation procedure was used.

Another attempt to design a method whereby apo and biotinylated 1.3 S proteins could be completely separated involved the use of hydrophobic interaction chromatography. Hydrophobic interaction chromatography (HIC) was performed as described under Materials and Methods on a Progell TSK Ether 5PW column. The sample, after avidin (monomeric)-agarose chromatography, was loaded in the presence of 2 M ammonium sulfate in 0.1 M potassium phosphate buffer, pH 6.8. The separation of the biotinylated and apo 1.3 S subunits was achieved by a linear negative gradient of 0.1 M potassium phosphate
Figure 16. Reverse phase HPLC of avidin purified 1.3 S protein

Avidin purified 1.3 S protein was applied to a SynchroPak RP-4 (4.6 mm x 25 cm). Elution was isocratic in solvent A for 10 min followed by a 2.5 min linear gradient (0-40% B), then isocratic at 40% B for 15 min followed by a 2.5 min linear gradient (40-100% B). Solvent A was 0.1% TFA in H$_2$O, solvent B 0.1% TFA in acetonitrile. Peak 1 is apo 1.3 S and peak 2 biotinated 1.3 S.
buffer, pH 6.8, containing 2 M ammonium sulfate (solvent A) and 0.1 M potassium phosphate buffer, pH 6.8 (solvent B). The elution profile showed two major, well resolved peaks as shown in Figure 17. In order to determine whether the apo and biotinylated 1.3 S subunits were separated completely by the above procedure, material in each of these two peaks was dialyzed against water and lyophilized. By SDS PAGE (Fig. 18), amino acid composition and N-terminal sequencing, both peaks were shown to be homogeneous 1.3 S. The biotin content of both peaks was estimated and the results are presented in Table I. Protein in peak 1 did not contain any biotin whereas the protein in peak 2 contained biotin in a biotin to protein ratio of 1:1, indicating that it contained only biotinylated 1.3 S subunit. Moreover, the fact that the biotinylated 1.3 S subunit and the apo 1.3 S subunit were separated by hydrophobic interaction chromatography suggest that the two forms have different hydrophobic characteristics. This difference most likely results from the presence or absence of biotin, itself a strongly hydrophobic molecule.

In order to demonstrate that the purified apo and biotinylated 1.3 S subunits can bind to avidin (monomeric)-agarose, each purified protein was loaded on an avidin (monomeric)-agarose column and eluted as described under Purification of 1.3 S subunits. Both forms of 1.3 S bound to the column and were eluted by biotin-containing buffer (data not shown) under our standard conditions for the purification of 1.3 S from crude extracts.
Figure 17. Hydrophobic interaction chromatography of avidin-purified 1.3 S protein

Avidin-purified 1.3 S protein was applied to a TSK Ether 5 PW HIC column (0.75 cm x 7.5 cm). Elution was isocratic in buffer A for 10 min. followed by a negative ammonium sulfate gradient of 2 - 0.8 M over 9 min. and isocratic in 0.8 M ammonium sulfate for 6 min. followed by another negative gradient of 0.8 - 0.0 M ammonium sulfate. Buffer A was 2 M ammonium sulfate in 0.1 M potassium phosphate buffer, pH 6.8 and buffer B was 0.1 M potassium phosphate, pH 6.8. Peak 1 was shown to be apo 1.3 S and peak 2 biotinyl 1.3 S.
Figure 18. SDS-PAGE analysis of HIC purified 1.3 S

Western analysis, with anti-TC antiserum, of 1.3 S proteins separated by 15% SDS-PAGE. Lane 1 - pre-stained molecular weight standards (phosphorylase b, 97,400; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400); lane 2 - avidin-purified 1.3 S; lane 3 - peak 1 from HIC HPLC; lane 4 - peak 2 from HIC HPLC.
The Effect of Heat Treatment on 1.3 S Mutants

To assess the potential of heat coagulation as a purification step, crude extract samples of 1.3 S WT were treated for ten minutes each at 55, 65, 75, 85, 95°C and the soluble fraction was analyzed by SDS-PAGE for purity and recovery and by reconstitution with 12 S and 5 S subunits for determination of TC overall reaction activity. Parallel experiments were carried out on purified 1.3 S in buffer and purified 1.3 S which was added to a crude extract from a negative control (host E. coli). Transcarboxylase reconstitutions were set up with a 12 S:5 S:1.3 S ratio of 1:6:12 based on 0.01 nmole 12 S with estimated 1.3 S concentrations in a final volume of 100 µl. These reconstitution reactions were assayed for activity in the overall transcarboxylase reaction after incubations of 1, 4 and 21 hours. Figure 19A shows the SDS-PAGE from this experiment and Figure 20 is a Western analysis of identical gels. Table II shows the 1.3 S activity after each treatment. The results indicate that the activity of the 1.3 S subunit is resistant to the heat treatments employed. Also, heat coagulation resulted in significant removal of soluble proteins from these extracts without any apparent loss of 1.3 S. Further, this was our first demonstration that the I94L mutant was as active as wild type 1.3 S.

The effect of crude extracts and heat treatment on assembly and overall reaction activity was assessed by reconstitution of transcarboxylase complexes and assay of overall transcarboxylase reaction activity. The effect of the presence of other bacterial proteins on activity in the 12 S and 5 S partial reactions was also determined. The ability of 1.3 S protein to assemble and catalyze the overall
Figure 19. Heat treatment of 1.3 S extracts and purified protein

1.3 S samples were treated for 10 min. at room temperature (R.T.), 55, 65, 75, 85, or 95 °C. After treatment, the samples were centrifuged to remove precipitate and analyzed by SDS-PAGE and TC complex reconstitution and activity measurement. Coomassie stained 15% SDS-PAGE gels shown here were: A - purified 1.3 S protein added to a negative control *E. coli* extract (pKK223-3/CH26); B - negative control *E. coli* extract (pKK223-3/CH26); C - 1.3 S expression extract (ptac1.3/CH26); D - pure 1.3 S (4 nmole/sample) in 10 mM potassium phosphate buffer, pH 6.8. Lanes are paired with 1 and 5 μl loaded in each case, the temperature treatment is indicated above each pair. Arrowheads on the right side indicate the 1.3 S band. Molecular weight standards (phosphorylase b, 97,400; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400) were run on each gel - A, lane 1; B - lane 3; C - lane 5; D - lane 7.

"
Figure 20. Heat treatment of 1.3 S extracts and purified protein

1.3 S samples were treated for 10 min. at room temperature (R.T.), 55, 65, 75, 85, or 95 °C. After treatment, the samples were centrifuged to remove precipitate and analyzed by SDS-PAGE and TC complex reconstitution and activity measurement. Western analyses of 15% SDS-PAGE gels shown here were: A - purified 1.3 S protein added to a negative control *E. coli* extract (pKK223-3/CSH26); B - negative control *E. coli* extract (pKK223-3/CSH26); C - 1.3 S expression extract (ptac1.3/CSH26); D - pure 1.3 S (4 nmole/sample) in 10 mM potassium phosphate buffer, pH 6.8. Lanes are paired with 1 and 5 µl loaded in each case, the temperature treatment is indicated above each pair. Arrowheads on the right side indicate the 1.3 S band. Molecular weight standards (phosphorylase b, 97,400; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400) were run on each gel - A, lane 1; B - lane 3; C - lane 5; D - lane 7.
Table II

Transcarboxylase overall reaction activity of heat treated 1.3 S protein samples

<table>
<thead>
<tr>
<th>1.3 S sample</th>
<th>TC activity (U/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unheated</td>
<td>65°C</td>
<td>85°C</td>
</tr>
<tr>
<td>pure 1.3 S</td>
<td>0.42</td>
<td>0.42</td>
<td>0.42</td>
</tr>
<tr>
<td>pKK223-3/CSH26 bacterial extract</td>
<td>0.05</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>1.3 S + pKK extract</td>
<td>0.50</td>
<td>0.40</td>
<td>0.48</td>
</tr>
<tr>
<td>ptac1.3/CSH26 bacterial extract</td>
<td>0.46</td>
<td>0.53</td>
<td>0.50</td>
</tr>
</tbody>
</table>
reaction was shown to be unaffected by heat treatment or the presence of contaminating proteins (Table II). Dr. Wood assayed the 12 S and 5 S partial reactions in crude extracts of bacteria expressing each of these recombinant proteins. Activity was also measured in the 12 S partial reaction with 30-60% ammonium sulfate fractions of these crude extracts. In each of these assays, the contaminating proteins did not interfere with the ability to measure partial reaction activity.

**Hinge Region Mutagenesis**

Most of our previous mutants were constructed by insertion of synthetic oligonucleotide cassettes into restriction digested plasmids as described (34). The putative hinge region of the 1.3 S gene does not have any restriction endonuclease recognition sites which could be used in this strategy. This characteristic of the region necessitates the use of primer-directed mutagenesis of ssDNA as described in Methods.

To analyze the function of the conserved -P-A-P- segment of the "hinge" region, mutagenic oligonucleotides were designed to replace both proline codons with codons for alanine, tryptophan or histidine. In each case, the mutation also produced a new restriction endonuclease recognition site which could be used in screening for putative mutants. The alanine substitution for proline was designed to be a drastic change which would minimize the rigidity of conformation of the hinge region. Conversely, the tryptophan substitution was conceived as the bulkiest side-chain possible which would thus provide maximal steric hindrance to motion around the peptide bonds of the hinge region without the specific
constraint characteristic of the proline residues. The histidine substitutions were considered as potentially useful probes of microenvironment in future NMR studies.

Our expression construct plac1.3S was constructed by insertion of the PstI/HindIII fragment of ptacl.3t into PstI/HindIII digested pTZ18. Expression of 1.3 S protein from the pTZ1.3 phagemid was detected by Western analysis and 14C-biotin incorporation in vivo.

Analysis from screening plaques resulting from mutagenesis reactions with either the 94 Sac primer (described in Chapter IV - construction of 1.3 S I94L) or the PPN1 primer (pro → ala, 58, 60) led to identification of phagemid with wild type insert or without any insert. When the mutagenesis was run in the absence of primers, the results were identical to the previous attempts. The fact that second strand synthesis was independent of added primer indicated that the template was contaminated by some form of DNA fragments which were capable of priming synthesis. To prepare clean template free of contaminating DNA fragments, several purification methods were attempted. Preparations of single-stranded phagemid DNA were further purified by nucleic acid affinity column (Qiagen), phenol and chloroform extraction, agarose gel purification, proteinase K and SDS treatment but all samples continued to self-prime.

After multiple attempts, including variations which had been successful in other labs which had used phagemid techniques, it became clear that our single-stranded phagemid DNA could not be adequately separated from single-stranded helper phage DNA or other polynucleotide fragments. Our insert was much smaller than the inserts used successfully in other laboratories and it was
concluded that the resulting size of our phagemid DNA was insufficient for adequate separation from contaminating DNA.

Bacteriophage M13 has been used extensively as a source of single-stranded DNA for sequencing and mutagenesis. M13 was chosen as a mechanism by which to avoid the problems encountered with the pTZ phagemid because it does not require a helper phage. Among the disadvantages of the M13 system was the fact that protein expression was so weak that, after construction of the mutation, new mutants required subcloning into an expression vector.

As with the pTZ18 subcloning, the bacteriophage vector M13mp18 was digested with Pst1 and HindIII for ligation with the Pst1/HindIII fragment of ptacl.3t. During attempts at this subcloning, two errors in ptacl.3t were discovered. Repeated attempts at this subcloning failed until an extra control was checked and a second Pst1 site was identified between the Pst1 and HindIII recognition sites. This second site was within ten bases of the HindIII site which is why it had not been identified previously. The presence of this second site accounted for the difficulty in subcloning and indicated that earlier subclones generated by this protocol were only accomplished when partially digested DNA allowed for compatible ends. These same analyses indicated that the ptacl.3t plasmid was a dimer. The correct Pst1 fragment was then subcloned into a fresh preparation of pKK223-3 and this plasmid, termed ptacl.3, was used in all subsequent experiments. The desired M13 subclone was identified by PCR with the M13 reverse sequencing primer and either the M13 17-mer universal primer or our 14-mer oligo 118CL (32). Expression of 1.3 S protein by E. coli cultures infected with M13-1.3 was detectable but was very low relative to our standard plasmid expression system.
At the same time as this shift from the phagemid mutagenesis strategy to the M13 system, it was also decided to broaden our mutagenesis. The peptide data (70) indicated that the presence of the glycine/valine repeats might be more critical than the conserved P-A-P segment since one peptide which functioned well did not contain the first proline. Rather than mutate each glycine or valine residue, it was decided that the repeats should be investigated through the use of deletion mutants. Figure 21 shows the segments to be deleted in each of the proposed mutants.

The first member of this series was designed to remove the coding sequence for residues 58-64 and generate a mutant termed 1.3 S Δ 58-64. This sequence represents the conserved P-A-P and the first G/V repeat.

The mutagenesis was carried out as described in Methods. Plaques resulting from the mutagenesis were selected at random and propagated in E. coli so that phage DNA could be analyzed. The identity of the clone was confirmed by restriction digestion and sequencing with two primers, one complementary to the 1.3 S gene and the other to the multiple cloning site of bacteriophage M13.

After identification of the desired mutant insert in M13, the insert was subcloned into pKK223-3 as a PstI fragment and transformed into E. coli strain CSH26 for expression of mutant protein. Subclones were subjected to restriction digestion with EcoRI and XhoI to verify both the identity of the insert and its orientation. Figure 22 shows the difference in electrophoretic mobility which resulted from the deletion.

To determine protein expression from the deletion construct, Western analyses and in vivo biotination were assessed. The deletion construct did not express any detectable protein by either technique. To attempt to improve the
Figure 21. 1.3 S "hinge" region deletion plan

The sequence is shown of the central segment of the 1.3 S subunit. This segment was the focus of interest after peptide studies identified a functional role for this region. Residues noted as conserved are shown in bold type. The boxed segments are identified as the proposed deletions.
GEGE

PAPLAGTVSKILVKEDTVKAGQTVLV

Δ 58-64   Δ 63-69   Δ 69-74   Δ 74-80
Figure 22. DNA mobility of ptac1.3(Δ58-64)

The PstI DNA fragment carrying the 1.3 S gene was run on a 5% TBE acrylamide gel to demonstrate the increased mobility of the gene carrying the deletion. Lane 1 is ΦX174, HaeIII DNA size standards, lane 2 is the 1.3 S Δ58-64 gene and lane 3 is the wild type 1.3 S gene fragment.
expression of this mutant we 1) resubcloned the insert into fresh vector; 2) subcloned into an alternate vector; 3) altered growth conditions through the use of different medias, growth temperatures, cell densities or induction conditions. The promoter in the initial subclone apparently was not deficient since fresh vector did not provide a measurable rate of expression. The choice of an alternate promoter also did not overcome the expression problem. Growth overnight at 37°C in extra rich media, only increased accumulation to a minimum detectable level (~20 nanograms), not enough to attempt purification. Accumulation of the deletion mutant was approximately 30-fold less than 1.3 S I94L which was about 10-fold less than wild type 1.3 S (Figure 23). With this failure, the entire deletion series was discontinued.

The C Terminal Fragment

Before the peptide data (70) were generated which described the lack of activity of 1.3 S fragment 78-123, truncated 1.3 S expression constructs had been made to study the ability of N terminally shortened biotinyl peptides to catalyze the transcarboxylase partial reactions. Protein expressed by the construct ptac1.3(85-123) could not be detected (84). Since this peptide continued to be of interest in our studies, it was decided to use a fusion vector to construct the 1.3 S fragment as an N terminal extension on β-galactosidase. The fusion vector, pJG200 expresses cloned fragments as the amino terminal segment of a fusion protein with β-galactosidase and a cleavable linker of a short collagen fragment.

As described in Methods, the fusion vector pJG200 requires the coding segment of the protein of interest to be inserted as an BamH1 fragment. To
Figure 23. Expression of 1.3 S Δ58-64

Expression extracts of *E. coli* CSH26 (lanes 1-3), ptac1.3(D58-64)/CSH26 (lanes 4-6), ptac1.3I94L/CSH26 (lanes 7-9), and ptac1.3/CSH26 (lanes 10,11) were separated on a 15% SDS-PAGE gel and analyzed by Western blotting for expression of 1.3 S immunoreactive protein. Each set of samples were loaded in increasing volume (2, 10 and 15 μl) of extracts standardized by culture turbidity. Lane 12 contained prestained molecular weight standards (phosphorylase b, 97,400; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400) in place of the ptac1.3 15 μl sample.
accomplish this, two new 1.3 S expression constructs were generated as cloning intermediates. The construction of a ptac1.3 vector whose coding segment could be subcloned as a BamH1 fragment was accomplished as described in Methods and resulted in a construct referred to as ptac1.3(1-123AP).

With ptac1.3(1-123AP) as the starting material, another construct was made, ptac1.3(85-123AP), by insertion of a start site for translation before the codon for amino acid 85. This construct thus carries the coding region of 85-123 on a BamHI restriction fragment.

Plasmid transformed *E. coli* were analyzed for accumulation of each of these new 1.3 S constructs by immunostaining and by *in vivo* biotination. The presence of the plasmid expressing 1.3 S (1-123AP) led to accumulation of significant amounts of immunoreactive 1.3 S protein. This strain accumulated approximately 30-50% of the level of protein seen with the wild type expression construct. The level of *in vivo* biotination as determined by incorporation of $^{14}$C-biotin was correspondingly decreased relative to wild type expression. The results of these studies of expression from ptac1.3(1-123AP) were reported by Murtif and Samols (34). Similar analyses of the ptac1.3(85-123AP) construct confirmed the results of Murtif *et al* with ptac1.3(85-123) - neither immunoreactive nor biotinylated protein could be detected.

Sequencing of these new 1.3 S constructs verified the presence of the desired inserts and their proper reading frame for subcloning to the fusion vector and identified a point mutation in the tac promoter which was responsible for the decreased expression found with all 1.3 S constructs made from ptac1.3(1-112). Protein expression by all constructs, except ptac1.3(85-123) and ptac1.3(85-
123AP), improved significantly after subcloning to a new batch of pKK223-3 with the wild type promoter.

A side product of the construction of ptac1.3(1-123AP) resulted in the production of ptac1.3("1-132"). The strategy used in the construction of ptac1.3(1-123AP) required that the synthetic oligonucleotide cassette have MluI sites at both ends; thus, the insert could ligate in either orientation. A reverse insertion of this cassette would encode a 1.3 S protein, termed "1-132", with the sequence: 1-112-LWIPDLDETLTALHSFCFGG. A clone of this construct was isolated and analyzed as above for the 1-123AP protein. Western analysis showed an immunoreactive protein slightly larger than the 1.3 S protein, but no biotination could be detected. Murtif et al (34) showed that a penultimate hydrophobic residue was required for in vivo biotination. This 132 residue protein has a leucine in the position corresponding to 122 but is not biotinylated. The rest of the 112-132 sequence is highly divergent from the wild type 1.3 S sequence and is most likely the cause of lost biotination.

The BamHI restriction fragments of ptac1.3(1-123AP) and ptac1.3(85-123AP) were subcloned into pJG200 to construct the 1.3 S-β-gal fusion vectors. Expression of the fusion constructs was assessed by Western analysis with both anti-TC and anti-β-galactosidase antisera. β-galactosidase activity was assessed by spectrophotometric assay to determine whether the fusion protein maintained β-gal activity. The fact that the BamHI fragment from ptac1.3(85-123AP) carried the tac promoter with it into the fusion vector was a concern in relation to its affect on protein expression. Since the fusion proteins were designed to be purified by affinity chromatography on a resin conjugated with anti-β-galactosidase antibodies, detection of the fusion protein by anti-β-galactosidase
antisera and the presence of β-galactosidase enzymatic activity were taken as indicators of the likelihood of success in purification of the expressed proteins.

Western analyses of several isolates of each fusion construct were conducted to assess the presence of expressed protein immunoreactive with the anti-1.3 S and the anti-β-galactosidase antisera. Several isolates could be identified as positive in each case, but the molecular weights varied and individual isolates were not consistently positive with both antisera. Activity assays of β-galactosidase activity in extracts from these isolates of the fusion constructs confirmed the Western data but did not provide any further confidence in the identification of the expressed proteins. Further, by [14C]biotin in vivo biotination assay, none of the clones showed any evidence for the presence of biotin.
DISCUSSION

Apo-1.3 S affinity for avidin-sepharose

Avidin- biotin technology has been used extensively in several areas of research such as the purification of biotin-containing carboxylases and proteins chemically modified by biotin attachment. The avidin-biotin system has become popular because of the exceptionally high affinity and stability of the avidin-biotin complexes. The affinity of native avidin toward biotin is so high that they form essentially irreversible complexes. Because of the high affinity of biotin toward avidin, the conjugate of native tetrameric avidin with biotin can be disrupted only under denaturing conditions. To reduce the affinity of the biotin toward avidin tetramer and to avoid denaturing conditions for isolation, avidin columns with lower affinity toward biotin have been constructed. These columns are made up of avidin-monomers instead of avidin tetramers; the affinity of monomers toward biotin is reduced from a $K_d$ of $10^{-15}$ M to $10^{-8}$ or $10^{-9}$ M ($59, 60, 85$). The elution of the bound biotinylated proteins can then be achieved by lowering the pH to 2.0 with glycine-HCl buffer or with a buffer containing biotin. A number of carboxylases such as pyruvate carboxylase ($64$) and acetyl-CoA carboxylase ($56$) have been purified using avidin (monomeric)-agarose columns in a one step affinity procedure.

In the present study we used avidin (monomeric)-agarose from Sigma for purification of the 1.3 S subunit expressed in $E. coli$. To our surprise we found that the column bound both apo and biotinylated 1.3 S subunits specifically. The
apo form was detected when the biotin content of the avidin (monomeric)-agarose purified material was determined. Instead of the expected one n mole of biotin per n mole of 1.3 S subunit, only 0.28 n mole of biotin was found. Thus, the material purified by the avidin (monomeric)-agarose column contained a considerable amount of apo 1.3 S subunit, which is further substantiated by the fact that it is a substrate for the synthetase. No other proteins bound to the avidin column as judged by SDS PAGE, amino acid composition and N-terminal sequencing of the protein eluted from the column. The bound apo and biotinylated 1.3 S subunits could be eluted either by using a biotin containing buffer or a glycine-HCl buffer, pH 2.0. Avidin columns with a mixture of apo and biotinylated 1.3 S protein bound were washed with buffers of various pHs or salt concentrations to disrupt protein-protein charge interactions. This treatment was ineffective in separating the biotinyl protein from its apo form (data not shown). A second possible mode of interaction, disulfide bonding, which could provide a mechanism by which apo protein would copurify with biotinylated 1.3 S was eliminated from consideration since 1.3 S does not contain any cysteine residues.

We used two different methods to separate the apo and biotinylated forms of the 1.3 S subunit. Both methods depended on the hydrophobicity of the proteins as the biotinylated 1.3 S subunit should be more hydrophobic than the apo form. In the first method, we employed reverse phase HPLC, where the separation mode is by interaction with total hydrophobic amino acids, using a C4 column with a TFA/acetonitrile gradient. Under these conditions, although the apo and biotinylated proteins were not well separated from each other, they could be distinguished indicating that this method was useful to rapidly assess the presence of apo contaminants in biotinylated 1.3 S subunit preparations. In order
to maximize the separation of the apo and biotinylated forms as well as to improve the conditions by maintaining the native state of the proteins, we developed an alternate procedure involving hydrophobic interaction chromatography using an ammonium sulfate gradient in phosphate buffer. This hydrophobic interaction chromatography separates the proteins in their native conformation and the separation mode is by interaction with only the surface or exposed hydrophobic amino acids. This method provided clear separation of the apo and biotinylated 1.3 S subunits. Further, the results from SDS PAGE, amino acid composition, N-terminal sequencing, biotin content and synthetase action indicate that the apo 1.3 S subunit elutes earlier than the more hydrophobic biotinylated protein.

Co-purification of the apo and biotinylated forms of 1.3 S could arise from strong protein-protein interactions between either (i) the apo and biotinylated 1.3 S subunits or (ii) the apo 1.3 S and avidin. Fluorescence quenching results indicated that both apo and biotinylated 1.3 S forms bind tetrameric avidin equivalently (81). Moreover, the purified apo form of the 1.3 S subunit bound as well as the biotinylated form to the avidin (monomeric)-agarose column. This result suggests that the co-purification of the apo 1.3 S subunit with the biotinylated 1.3 S subunit was not due to protein-protein interactions between apo and biotinylated subunits. It is possible that both apo and biotinylated 1.3 S bound to avidin at avidin's biotin binding site so that both were eluted by biotin due to a competitive displacement. Alternatively, both apo and biotinylated 1.3 S may bind to avidin at a site other than the biotin binding site and may co-elute as a result of a conformational change in avidin caused by biotin binding. However, it has been shown by Green (85) and Kurzban et al (86) that binding of biotin to
avidin does not cause any gross conformational changes, making the latter possibility less likely. As the biotin-containing buffer efficiently eluted the apo form of the 1.3 S subunit, the most likely interpretation of our data is that the interaction of avidin with the two 1.3 S forms was not through biotin but at another site on the 1.3 S protein. The carbohydrate moiety conjugated to avidin has been implicated as a factor causing non-specific interactions (62). Considering that apo and biotinylated 1.3 S subunits both interact tightly with avidin and are specifically eluted by biotin, it is likely that they both bind at the "biotin-binding site" of avidin. These results would suggest that this "biotin-binding site" recognizes a peptide folding feature of the 1.3 S subunit other than or in addition to biotin itself. It is possible that the folded state of this putative avidin binding region of the 1.3 S subunit may mimic the chemical environment of biotin molecule per se. Currently, investigations are underway to determine the regions of the 1.3 S subunit which bind to avidin specifically.

Our results suggest that the avidin-biotin technology by itself may not be sufficient for one step purification of homogeneous biotinyl proteins. Non-biotinyl peptides can also bind making further purification by other methods necessary. Moreover, our study indicates that hydrophobic interaction chromatography may be used for the separation of apo and biotinylated forms of proteins. Beyond taking this data as a caution in the use of avidin affinity purification, it should be taken as a commentary on the problems hidden in the growing popularity of avidin-biotin reagents.

Another example of the problems which may be caused by incomplete understanding of avidin-biotin interactions when using this technology as a reagent, a group at the University of Toronto has reported the unintentional
cloning of a biotinyl protein. Sandham et al. (87) used specific oligonucleotides with biotin labels and an alkaline phosphatase-linked avidin detection system to screen a *Streptococcus mutans* expression library. After selection and purification of a positive clone from their expression library, they demonstrated that their clone does not bind the oligonucleotide but a database search with their sequence indicated that they had cloned a biotinyl protein. The avidin conjugate whose use was intended to detect the biotinylated oligonucleotide specifically bound to an appropriate plaque recognized the biotinyl protein in their lysed colonies and led them to select a clone which was expressing a biotinyl protein rather than one which hybridized with their oligo probe. Had this type of false positive been considered in advance, the problem could have been avoided by use of a different type of library or by a simple control of hybridization with the second reagent in the absence of the labelled oligo. However, these consequences of these new technologies may be an advantage to researchers interested in the biotinyl proteins. Dr. Sandham was kind enough to provide a sample of their bacteria for our analysis. A *Streptococcus mutans* culture was grown anaerobically in LB media, lysed by lysozyme treatment and analyzed by Western analysis with anti-TC antiserum and transcarboxylase overall reaction activity assay. The biotinyl protein of *S. mutans* was immunoreactive with our anti-TC antiserum but did not show any activity. Comparison of the sequences indicated that they are 33% identical and 56% similar. With this striking level of similarity, cross-reactivity of the antisera was not surprising.

Wilchek and Bayer (62) have shown that avidin will recognize the RDL fibronectin sequence, binds it specifically, and that this binding is enhanced by first binding biotin to the avidin. The observation that biotin enhances the binding of
avidin to this fibronectin sequence implies the presence of a second binding site and may call into question earlier statements by Green that biotin binding does not cause a conformational change in the avidin. The fibronectin binding site on avidin would be a candidate for identification as the 1.3 S binding site except that fibronectin binding is enhanced by biotin while both apo and biotinyl 1.3 S can be released by the presence of biotin. This observation does, however, indicate another caution in the use of avidin detection systems - in addition to a background of biotinyl proteins, other proteins may also interfere.

Having recognized that avidin may bind to unexpected or unintended molecules, it is also important to understand what molecules are capable of binding biotin. Avidin and streptavidin are the commonly recognized biotin-binding proteins, but it should be considered that the biotin operon repressor (88), holocarboxylase synthetase (89) and biotin carboxylase subunits of the biotin dependent carboxylases (6) are also capable of binding free biotin and thus likely to be capable of binding biotinylated probes used in many new applications. Recombinant phage libraries expressing random peptides have recently come into use in screening for active or reactive peptides (90). Saggio and Laufer recently used such a recombinant system to identify biotin-binding peptides (91). The consensus biotin-binding motif which emerged from their cloned peptides, CXWXPPF(KorR)XXC, is not found in any of the proteins known to bind biotin and may represent another unknown hazard to indiscriminate users of avidin-biotin technology.

The stability of the 1.3 S protein
Previous work showed that the 1.3 S subunit of transcarboxylase to be a small protein lacking in stable, discernible secondary structure which could be isolated in an active form despite treatment with harsh solvents. These characteristics led to consideration of heat treatment of expression extracts as a preliminary purification procedure. It was shown that heat treatment led to several fold enrichment of 1.3 S protein through removal of other proteins by heat coagulation. The activity of the 1.3 S protein, purified and in the form of an expression extract, was shown to be resistant to heat treatments from 55°C to 95°C. Further, it has been shown that contaminating E. coli proteins do not interfere in the specific assembly of transcarboxylase complexes or in the assay of their activity.

The 1.3 S "hinge" region

The region of the 1.3 S sequence from amino acid 58 to 78 has drawn interest as a putative hinge region which may be involved in the movement of the biotinyl moiety of the 1.3 S subunit between the active sites on the other two transcarboxylase subunits. Peptide data and sequence conservation led to the targeting of the conserved P-A-P and G/V residues for functional analysis. Our mutagenic studies have not extended these data except to demonstrate that there is a characteristic of residues 58-63 which contribute to the stability of the protein. It would be interesting to know if deletion of the other G/V repeats would have the same destabilizing affect on the protein. Beyond this, single residue mutation, like the originally planned proline substitutions, will be needed to determine the function of these conserved residues.
Recent analyses by Toh et al (92) and Brocklehurst and Perham (67) have lent support to the concept of the 1.3 S subunit, and its evolutionary relatives, folding in such a way as to provide a flexible arm with the prosthetic group positioned as the active "hand" at the end of the "arm". The proline, glycine and valine residues discussed above all are conserved as demonstrated by the sequence homology between biotinyl and lipoil proteins. Solution of the 3-dimensional structures of the pyruvate dehydrogenase lipoil domains of Bacillus stearothermophilus (68,69) and E. coli (93) by NMR spectroscopy allowed these analyses of sequence homology to be placed in a structural context. Further, the 3-dimensional structure indicates a β sheet framework for this "flexible arm". These data fit well with a model proposed by Kumar et al (35) in which the biotin movement is directed by a structural shift between α helix and β sheet in critical portions of the protein. In this model, the biotin is positioned at the end of a flexible arm and surrounded by residues critical for interaction with active sites. Importantly, this flexible arm does not move randomly between active sites with an infinite number of possible positions, but rather, has two positions and is switched between these positions by an element in the arm which can fold as an α helix or β sheet depending on the modification condition of the prosthetic group. Thus, an uncarboxylated subunit's "switch segment" might be folded as a β sheet and, upon carboxylation, switch to an α helical conformation with this switch moving the biotin from one active site to the other.

The Perham group has recently determined the 3-dimensional structure of the biotinyl domain of E. coli acetyl-CoA carboxylase (94) and found it to be nearly indistinguishable from their previously determined lipoil domain structure. This determination verified the positioning of the biotin and the flexibility of its
protein domain. Further, it was apparent from this structure that the glycine residues noted through sequence conservation play roles in the flexibility of the domain while the valine residues function in anchoring the beta sheets to the domain's hydrophobic core. The conserved prolines occur in beta turns which connect the eight beta sheets of the domain.

The 1.3 S C terminal region

The fusion protein expression experiments reported here were designed to provide us with material with which we could assess the functionality of a protein representing only the C terminal region of the 1.3 S protein. Our interests were in the ability of holocarboxylase synthetase to recognize this truncated form for biotinylation, interactions of this peptide with the other subunits of TC and function in the partial reactions of TC.

Our approach to these studies proved to have more obstacles than expected and was not carried to completion. While the cloning was accomplished, analyses of extracts of the putative clones gave inconsistent results when analyzed for immunoreactive protein. One consistent result was the lack of evidence for biotination in any of the constructs analyzed. Apparently, the bulk of the β-galactosidase protein fused to the C terminus of the 1.3 S protein or peptide masked it from recognition by holocarboxylase synthetase. This fact alone prevented analysis of the ability of the C terminal peptide to function as a substrate for biotination in vivo. If the immunological analyses had been consistent, purification of the fusion proteins should have allowed isolation of the 1.3 S protein and the C terminal peptide for in vitro study with purified
holocarboxylase synthetase and, if \textit{in vitro} biotination had been successful, study of activity in the transcarboxylase partial reactions.

Cronan, working with our ptac1.3t plasmid, generated a series of fusion constructs with different lengths of the 1.3 S protein \textsuperscript{(33)}. The expressed fusion proteins contained the 1.3 S fragments fused to the carboxyl terminus of \(\beta\)-galactosidase. The truncated constructs used in this study defined the C terminal 60-74 residues of 1.3 S as the minimum peptide functional for biotination \textit{in vivo} through the action of holocarboxylase synthetase. This expression system has since been exploited by Promega and is marketed as the PinPoint\textsuperscript{TM} expression and purification system. The Promega kit provides a modified ptac1.3t plasmid which produces amino terminal fusion of the entire 1.3 S protein through a short, cleavable linker peptide. The resultant fusion protein is N terminally labeled with the biotinyl 1.3 S tag and is purified by chromatography on a monomeric avidin affinity resin from Rohm and Haas Company.

It is not known why biotination could not be detected in the pJG200 fusion construct containing the 1.3 S gene while Promega has achieved reliable expression and biotination with a very similar vector. The chief differences between these vectors are the linker peptides and the promoters. The reason for the success or failure of this technique to be dependent on these features is not apparent.
CHAPTER IV

1.3 S TETRAPEPTIDE REGION

This work contributed to the following publications:


INTRODUCTION

Sequence analysis of the 1.3 S subunit in comparison with the biotinyl subunits of six other biotin dependent carboxylases representing 11 species showed a startling level of sequence identity. One region indicated by this analysis surrounds the biotinyl lysine (position 89 in the 1.3 S sequence) and has been termed the conserved tetrapeptide. This tetrapeptide sequence, AMKM, with its symmetric methionines flanking the biocyitin, has been the subject of extensive site-directed mutagenesis (95-97). This conserved biotinyl tetrapeptide has been hypothesized as potentially significant in directing appropriate biotinylation by holocarboxylase
synthetase, presentation for biotin removal by biotinidase or for function in the
carboxylation or activity of the biotin (5).

Craft et al ruled out a function in association with biotinidase by
demonstrating that biotinyl 1.3 S is not a substrate for biotinidase (98). It was
shown that mutation of A87, M88 and M90 did not affect biotinylation of the 1.3
S subunit by E. coli holocarboxylase synthetase (95).

Having eliminated potential roles in the activities of holocarboxylase
synthetase and biotinidase, the various aspects of transcarboxylase activity were
examined for affects of tetrapeptide mutations. Those same 1.3 S mutants
examined in studies of biotinylation were shown to assemble full transcarboxylase
complexes but to be impaired in catalysis of the overall reaction (97). Attempts
were made to correlate the decreased activity of mutants with their ability to
associate with 12 S, 5 S or avidin. While some differences were detected by
fluorescence analyses, they did not correlate with subunit activity (96). Further
attempts, by circular dichroism spectroscopy and protease sensitivity, to identify
structural alterations which might explain the loss of activity in the 1.3 S mutants
indicated that the structure of 1.3 S M88L was slightly different from M90L and
WT, which were indistinguishable.

The impairment of activity in the three tetrapeptide mutants most
thoroughly studied, A87G, M88L and M90L, was shown to be a result of
decreased $k_{\text{cat}}$ while $K_M$ was unaffected (97). These studies found that the
mutations of residues 87 and 88 caused 5-10 fold decreases in $k_{\text{cat}}$ while the
mutation at 90 resulted in only a 20% decrease. Activity in the partial reactions of
subunit pairs indicated that mutation at methionine 88 had more dramatic effects
on activity than mutation of methionine at position 90. Similar conclusions were
drawn from assays of the partial reaction activities in transcarboxylase complexes, although the decrease in activity was less severe (96,99). Further studies of the partial reactions of the TC complexes (99) indicated that the transcarboxylation activity was affected more than the biotin carboxylation activity.

These studies led to the conclusion that residues 87 and 88 were critical to the correct conformation essential for biotin interaction at the active sites and catalysis of the transcarboxylase reactions. The facts that all of the mutations studied retained significant activity and were capable of stable assembly despite some structural differences led to the conclusion that these conserved residues are important for the proper conformation of the biocytin region but are not involved in catalysis. Further, the indication that these mutations preferentially affected the transcarboxylation reaction led to the suggestion that the stability of the carboxybiotinyl intermediate might be affected in the mutants (99).

This analysis has been limited because, although we have two or three amino acid substitutions at positions 87 and 88, we have only a single met \(\rightarrow\) leu substitution at position 90. To analyze the contributions of these two methionine residues (positions 88 and 90) relative to each other, mutations were designed to make equivalent changes to methionine 88 and to methionine 90. One set of paired mutants, 1.3 S M88L and 1.3 S M90L, had been constructed by Vicki Murtif (95,97). Two new sets were obtained by construction of 1.3 S M90C and 1.3 S M90T which are complementary to the existing mutants 1.3 S M88C and 1.3 S M88T. These three pairs of symmetric methionine mutants, cys, thr, and leu, were assayed in TC complexes for transcarboxylase activity, partial reaction activity and half-life of the carboxybiotin bond. The results of these studies and
analysis of the apparent activation energy of the carboxybiotin bond in each mutant and in the wild type context are presented here.
MATERIALS AND METHODS

Molecular biology

The source and handling of oligonucleotides was the same as described in Chapter III Materials and Methods. Other molecular biology methods were as described in Chapter III unless stated below.

The oligonucleotide sequences and their use in construction of new 1.3 S mutants is described below. The "94Sac" mutagenic oligonucleotide: GAGACCGAGCTCAACGCTCC was used in primer-directed site-specific mutagenesis of M13-1.3S ssDNA in production of a 1.3 S construct, ptac1.3 I94L, with a new restriction endonuclease recognition site (SacI) in the coding sequence of amino acid 94. This mutation also resulted in the conservative change of isoleucine 94 to leucine. When the mutant gene was subcloned from M13 to pKK223-3, the new restriction site was used as a diagnostic tool to verify the presence of the desired mutation.

The following oligonucleotides are shown as complementary pairs. After phosphorylation, each pair was annealed to form a mutagenic dsDNA cassette for insertion by ligation as described in Chapter III. These cassettes were ligated with XhoI, SacI digested, phosphatased ptac1.3 I94L DNA in low gelling temperature agarose.

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<tr>
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<th>TCGAGGCATGAAGACGGAGACCGAGCT</th>
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<tr>
<td>M90T-N</td>
<td>TCGAGGCATGAAGACGGAGACCGAGCT</td>
</tr>
<tr>
<td>M90T-C</td>
<td>CGGTCTCCGTCTTCATGGCC</td>
</tr>
<tr>
<td>M90C-N</td>
<td>TCGAGGCATGAAGGTGCAGACCAGCT</td>
</tr>
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</table>
M90C-C: CGGTCTCGCACTTCATGGCC

The products of these mutagenesis reactions were analyzed by PstI and BstXI restriction digestions - both mutations destroyed the BstXI site, thus providing a diagnostic reaction.

Purification of 1.3 S and mutant proteins

Purification of 1.3 S wild type and mutant proteins were accomplished as described in Chapter III except that 2 mM DTT was maintained in all buffers for purification of M88C and M90C.

Propionyl-CoA carboxylase purification and activity assays

The natural substrate for transcarboxylase is methylmalonyl-CoA. The 12 S partial reaction transfers the carboxyl group at the 3- position of the substrate to the biotin of the 1.3 S subunit. Assays of this partial reaction rely on the availability of [3-14C]-methylmalonyl-CoA. Malonyl-CoA and methylmalonyl-CoA are commercially available, but [3-14C]-methylmalonyl-CoA is not.

Propionyl-CoA carboxylase catalyzes the reverse of the 12 S partial reaction: \( \text{CH}_3\text{CH}_2\text{COSCoA} + \text{HCO}_3^- \rightarrow \text{CH}_3\text{CH}((\text{COO})\text{COSCoA} + \text{H}_2\text{O} \).

Thus, through the use of this enzyme and 14C-bicarbonate, the specifically radiolabeled substrate can be synthesized.

To use this reaction to produce the needed substrate, the propionyl-CoA carboxylase first had to be purified. According to the procedure of Gravel et al (65), 16 kg of fresh bovine liver were homogenized in Waring blenders. The
homogenate was made 20% carbon tetrachloride to precipitate membrane components, nucleic acids and some proteins. This solution was centrifuged, 5000xg for 15 min., to remove debris and organic precipitate induced by the solvent. After a second organic extraction, the supernatant was stirred overnight in the cold. Further precipitate resulting from this incubation was removed by centrifugation before application to avidin affinity columns. Portions of the supernatant which could not immediately be applied to the affinity resin were frozen at -20°C. After the samples were applied to the avidin columns, the columns were washed thoroughly with potassium citrate buffer. Elution of bound material was accomplished with potassium citrate buffer containing biotin. The eluted samples from the first run of the avidin columns were pooled and concentrated. This concentrated sample was assayed for activity, protein content and purity.

Propionyl-CoA carboxylase activity was assayed by incubation of sample aliquots with propionyl-CoA, 14C-bicarbonate, ATP and magnesium for one hour at 37°C. The reaction was stopped by addition of trichloroacetic acid. This acid treatment causes loss of 14C not incorporated into acid stable methylmalonyl-CoA. After acid treatment, the samples were dried, resuspended in water and quantitated in a scintillation counter with 963 scintillation cocktail. Specific activity can be determined by correction of counts according to paired controls and standardization of counts per minute based on the specific activity of the 14C-bicarbonate with final expression according to protein content. 1 unit of Propionyl-CoA carboxylase activity is the amount of protein which catalyzes the fixation of 1 μmole of [14C]-bicarbonate per minute at 37°C (65).
Assessment of activity and purity of the propionyl-CoA carboxylase in the purified samples indicated problems with the procedure. The sample had good activity, 0.42 mU/μg purified vs. 0.02 mU/μg crude, but that activity represented only 2% of the activity loaded on the columns. Further, the sample was highly contaminated by other proteins. Pure enzyme is not critical for preparation of the methylmalonyl-CoA, so it was decided to continue the procedure with the remaining extract.

The portions of the extract which had been frozen displayed aberrant behavior when purification was continued. Further precipitate, apparent when the second portion of extract was thawed, was removed by centrifugation. Supernatants were then applied to the regenerated avidin columns. However, as the extract was loading, it also continued to precipitate. The columns became clogged by precipitate and the precipitate could not be completely removed by incubation of the column resin in 0.2 M glycine-HCl, pH 2.0 or by incubation in urea.

Synthesis of [3-14C]-methylmalonyl-CoA was attempted with the partially purified propionyl-CoA carboxylase sample but was found to be inadequate for carboxylation of transcarboxylase complexes. It was then decided to accomplish the carboxylation through the use of partial reaction 2 and [4-14C]-oxalacetate.

Synthesis of [4-14C] oxalacetate

To 0.12 units of carboxytransphosphorylase (100) (purified by H. G. Wood and B. C. Shenoy and generously provided by Dr. Shenoy) that had been incubated for 15 min. at room temperature in 40 μl of 50 mM potassium
phosphate buffer, pH 6.5 with 1 μl of 100 mM DTT (total volume, 50 μl) was added 3 μl of 1 M phosphate buffer (pH 6.5), 7 μl of 100 mM MgCl₂, 10 μl of 300 mM KH¹⁴CO₃ (6,000 cp/mnmol) and 25 μl of 20 mM P-enolpyruvate. The formation of oxalacetate was monitored spectrophotometrically by reacting 5 μl aliquots with malate dehydrogenase. The reaction was usually complete in 20 min. and was stopped by addition of an equal volume of 0.1 N HCl. The oxalacetate content of the acidified solution was determined spectrophotometrically using malate dehydrogenase. The specific radioactivity was determined by addition of 5 μl of newly synthesized [4-¹⁴C] oxalacetate to 40 μl of 100 mM potassium phosphate buffer, pH 6.5, followed by 25 μl of reducing mix. The reducing mix contained per 25 μl, 2.4 units of malate dehydrogenase; 2.4 units of lactate dehydrogenase; 20 μg of NADH and 1.5 mmol of KHCO₃. After 5 min. at room temperature, 50 μl of 1 N HCl was added and the solution was evaporated to dryness under vacuum at 45°C. The dried samples were dissolved in 200 μl of H₂O and, after 5 min. (with occasional shaking), 2.8 ml of scintillant was added and the radioactivity determined. Usually about 0.3 μmol of [4-¹⁴C] oxalacetate was obtained and used without further purification.

Preparation of transcarboxylase complexes

Purified subunits were mixed in a 1.3:S:5 S:12 S molar ratio of 12:6:1 in 500 mM potassium phosphate buffer, pH 6.5 and incubated at 0°C overnight. Reconstituted complexes were purified from uncomplexed proteins by gel filtration HPLC on a ULTROPACK TSK SW4000G 7.5 x 300 mm column. The
elution profile clearly showed TC complexes, most likely 26 S and 18 S separated from free subunits. These peaks were collected and the fractions assayed for transcarboxylase overall reaction activity. Fractions with high activity were pooled and activity was determined for the pool. The protein concentration of the purified complex was determined by UV absorbance (101,102). Purified P. shermanii authentic 5 S was generously provided by Dr. K. Ganesh Kumar and 12 S protein purified from E. coli containing the plac 12 S expression plasmid was generously provided by Dan Sha.

$[^{14}\text{C}]$-carboxylation of transcarboxylase complexes

Carboxylation with the radiolabel was accomplished by incubation of TC complexes with $[4-{^{14}}\text{C}]$ oxalacetate for ten minutes at $15^\circ\text{C}$ followed by passage through a BioRad 10DG desalting column to remove substrates from the radiolabeled complex. Elution of the $^{14}$C-carboxybiotinyl TC complexes was followed by scintillation counting of 50 μl aliquots of each 200 μl fraction collected during elution. In every case, the radioactive profile demonstrated clear separation of the carboxylated complex from substrate. After pooling appropriate fractions, the $^{14}$C-carboxybiotinyl TC complex had to be handled quickly for determination of partial reaction activity and carboxybiotin stability. The sample was gassed with nitrogen, in the presence of a trace of Antifoam, continuously during the experiment so that $^{14}$CO$_2$ in solution would not interfere with measurement of radioactivity. Protein concentration of the complex was determined spectrophotometrically before assays began, but assay of biotin
content was carried out on an aliquot after all other determinations had been made.

Measurement of carboxybiotin halflife

Desalted $^{14}$C-biotinyl-TC complexes were incubated on ice for 10 min. with nitrogen bubbling at a rate of 240 ml/min. in the presence of 12.5 µg of carbonic anhydrase (provided by Dr. Shenoy) to remove $^{14}$CO$_2$ from solution. After this N$_2$ saturation, aliquots were separated for determination of the carboxybiotin stability at 0, 15, and 25°C. During the time courses, nitrogen continued to be bubbled and 75 µl aliquots were removed for immediate determination of radioactivity by scintillation counting. At the zero time point of each experiment, an aliquot was removed, treated with 50 µl of 6 N HCl and dried with heat (45°C) and vacuum to determine background radioactivity. In each experiment, radioactivity (cpm-background cpm) was plotted against time and analyzed for $t_{1/2}$ by the non-linear curve-fitting program NFIT.

Kinetic Analysis of Data

The non-linear curve-fitting program NFIT was used to fit the halflife data to the equation $y = Ae^{-kt}$. In this equation, $k$ is the rate constant which defines the halflife. The same program was used to transform these $k$ values for Arrhenius plots and determination of the apparent activation energy, $E_a$, with the equation $\log k = -E_a(1/2.3R)(1/T) + \log A$. 
RESULTS

Construction of mutations in the tetrapeptide region had concentrated on A87 and M88 because of the availability of the BstX1 restriction endonuclease recognition site which allowed relatively simple cloning of synthetic oligonucleotide cassettes designed to replace the wild type coding region from amino acid 85 to residue 90. Only one mutation had been made at M90 and no mutations existed in the conserved E91. The mutant 1.3 S I94L was designed in order to introduce a unique SacI restriction enzyme site at residue 94 facilitating the construction of mutants at positions 90-93. This introduction required the alteration of a single nucleotide and, in addition to the restriction site, resulted in the change of the encoded amino acid from isoleucine to leucine.

The construction of the 1.3 S I94L mutant was carried out in the bacteriophage M13 as described for the deletion mutant of 1.3 S. To identify the 1.3 S I94L construct, digestion with the restriction endonuclease SacI, whose recognition site was created by this mutation, was analyzed. After screening candidate plaques by this technique, the putative mutant was verified by DNA sequencing.

Since 1.3 S protein expression and accumulation were poor in M13 infected bacteria, the mutant gene was subcloned into the pKK223-3 expression vector, generating ptac1.3 I94L, and transformed into E. coli CSH26, the standard expression system for 1.3 S recombinant proteins. As 1.3 S I94L was the starting material for subsequent changes at other positions, its activity was compared to that of 1.3 S WT in initial control experiments. I94L mutant protein
accumulation was studied under a variety of conditions and shown to be significantly reduced from the level of expression of the wild type protein. This reduction in expression was shown not to be a function of a secondary mutation by further subcloning into pKK223-3 and pKK233-2 expression vectors without achieving any increase in expression. While protein accumulation was decreased by about 40%, with a similar reduction of in vivo biotination, in this mutant, it was still quite high and more than adequate for purification purposes.

Activity of 1.3 S I94L in partial and overall reactions

ptac1.3 I94L bacterial cultures were lysed and the expressed 1.3 S I94L protein in the partially purified dialyzed 30-60% ammonium sulfate fraction was assayed in the 12 S partial reaction by Dr. Wood. This was the first demonstration that crude extracts containing 1.3 S protein could be used in this partial reaction assay and also demonstrated that 1.3 S I94L was active.

The effect of heat on 1.3 S was studied to assess its utility as an initial purification step. Heat treatment was shown to remove the majority of bacterial proteins without precipitating 1.3 S or altering its activity. There was still a concern that 1.3 S mutants might behave differently than wild type under the heat stress. In response to this concern, wild type 1.3 S and 1.3 I94L were used for comparative studies of the effect of heat on crude extracts and the effect of heat on the ability of 1.3 S proteins to reconstitute TC complexes and catalyze the overall reaction as described previously.

The results indicated that the activity of the wild type and mutant 1.3 S proteins was quite resistant to heat. Also, heat coagulation resulted in significant
removal of soluble proteins from these extracts without any loss of 1.3 S, Figure 24. Further, this was our first demonstration that the I94L mutant was as active as wild type 1.3 S.

1.3 S M90T and 1.3 S M90C

The ptacl.3 I94L construct was created to facilitate further mutagenesis of the biotin tetrapeptide region, especially of methionine 90. To generate symmetric, paired mutations of methionine 88 and 90, the mutants 1.3 S M90T and 1.3 S M90C were designed.

The M90T and M90C mutations were accomplished with complementary oligonucleotides annealed to form double-stranded DNA cassettes encoding the desired region with upstream overhangs complementary to an XhoI site and downstream ends complementary to SacI overhangs. The sequences of the mutagenic oligonucleotides and the mutagenic protocol are described in Methods. One complicating feature of this strategy is that the ends of cassettes designed for this mutagenesis are complementary to each other. This adds potential undesired products to the mutagenesis reaction and requires that the screening strategy consider these other possibilities. The simplest side product is that the cassette
Figure 24. Heat treatment of 1.3 I94L

An expression extract of ptac1.3 I94L/CSH26 was treated for 10 min. at room temperature or at 75°C, cleared of precipitate and analyzed by SDS-PAGE and TC activity. The 15% acrylamide gel shown above was stained with Coomassie and dried. The unheated sample was loaded in lanes 1 and 2, 0.5 and 5 µl respectively. Similarly, the 75°C sample was loaded in lanes 3 and 4 with purified 1.3 S standard in lane 5 and molecular weight standards in lane 6.
may insert in the opposite orientation from that desired.

To identify these new constructs, DNA preparations from putative mutants were screened for loss of the BstX1 restriction site. Restriction digestion analyses identified the correct isolates of the new mutants 1.3 S M90T and M90C which were further verified by DNA sequencing.

Both plasmids were introduced into *E. coli* strains CSH26 and JM109 and analyzed by Western analysis for expression of immunoreactive 1.3 S protein. Both strains showed significant levels of accumulation of the mutants relative to I94L but, as expected, a lower level of accumulation than with the wild type construct. Immunoreactive protein levels were lower in JM109 than in CSH26. Biotination of the new mutants was assessed *in vivo* with $^{14}$C-biotin and found to be comparable for M90T and M90C both of which were reduced from I94L and wild type (see Figure 25).

Despite their biotin content, when attempts were made to purify the expressed proteins as previously described they did not bind well to the avidin-agarose affinity column. This was shown to be a characteristic of the proteins, not of the column or any other purification artefact. When extracts containing 1.3 S M90T or M90C were applied to the affinity columns by our standard methods, the bulk of the immunoreactive protein was found in the flowthrough. Elution of bound material allowed recovery of some mutant protein which had bound the columns, but it was contaminated with significant amounts of non-biotinyl bacterial proteins. It was shown that the biotin content of the load, the flowthrough and the eluted samples were all indistinguishable, thus eliminating the possibility that the columns were binding the biotinylated mutant while allowing the apo population to flowthrough. The columns were shown to be functioning
Figure 25. Expression and $^{14}\text{C}]$biotin incorporation in ptac1.3 M90T and ptac1.3 M90C

A. Phosphorimager analysis of $^{14}\text{C}]$biotin incorporation

*In vivo* biotination cultures and extracts were prepared as in Methods and analyzed by 15% SDS-PAGE. The samples were loaded in duplicate - lanes 1 & 2, negative control extract (CSH26); lanes 3 & 4, ptac1.3 I94L/CSH26; lanes 5 & 6, ptac1.3/CSH26; lanes 7 & 8, ptac1.3 M90T/CSH26; lanes 9 & 10, ptac1.3 M90C/CSH26.

B. Western analysis of 1.3 S expression

The same cultures as in A were analyzed for immunoreactive 1.3 S proteins. Lane 11, ptac1.3 I94L/CSH26; lane 12, ptac1.3/CSH26; lanes 13 & 14, ptac1.3 M90T/CSH26; lanes 15 & 16, ptac1.3 M90C/CSH26.
well by following an unsuccessful mutant purification attempt with a load of wild
type extract. Even after failing to adequately bind 1.3 S M90T or 1.3 S M90C,
the same columns bound the wild type protein normally. This comparison of
affinity toward 1.3 S proteins was carried out with both 1.3 S M90T and 1.3 S
M90C with comparable results. While there were purification problems with
these two mutants, wild type 1.3 S was readily purified by this same procedure.
The same observations were made with the 1.3 S mutants I94L, M88T and
M88C.

1.3 S M90T and 1.3 S M90C were successfully purified away from the
majority of contaminating proteins by a single passage through a C4 reverse phase
HPLC column. The difference in purity attained by reverse phase HPLC with or
without prior affinity chromatography of these mutants was not significant. For
preparative purposes, therefore, M90T and M90C were purified directly on C4
reverse phase HPLC. Other 1.3 S mutant and wildtype proteins were readily
purified as previously described. Purified 1.3 S wild type and mutant proteins
were assayed for protein concentration by rose bengal protein assay, purity as
judged by SDS-PAGE and biotin content by the Rylatt assay as previously
described. Contaminating proteins remaining after purification of some of the
mutants were shown not to interfere with complex assembly and were not present
in isolated complexes assembled from even the least pure 1.3 S protein.

In a preliminary study, transcarboxylase complexes were assembled from
reverse phase purified samples of wild type, I94L, M90L, M90T, and M90C 1.3 S
proteins. Assembly reactions were set up at a 12S:5S:1.3S subunit ratio of 1:6:18
with the 1.3 S concentration based on biotin determination to correct for various
degrees of purity. Overall reaction activity was assayed at 6 and 24 hours.
Complexes assembled with wild type 1.3 S, 1.3 S I94L or 1.3 S M90L were fully active while 1.3 S M90T had 20% of wild type activity and 1.3 S M90C displayed 10% of wild type activity. These assays were carried out in crude reconstitution mixtures with activity standardized to 12 S content of the mixture and were relevant in demonstrating that the new mutants were active and probably different from wild type.

**TC Complex Assembly, Activity and Biotin Content**

To more quantitatively assess the effect of each mutation on transcarboxylase activity, complexes were reconstituted from purified subunits, separated from free subunits by HPLC gel filtration and assayed for activity in the overall TC reaction. The 1.3 S mutants M90L and M88L were provided by Dr. Bhami C. Shenoy. 12 S subunit was purified by Dan Sha. The 5 S subunit used in these reconstitutions represented various preparations and was generously provided by Drs. G. K. Kumar and B. C. Shenoy. Prior to use in assembly, the 5 S samples were assayed for assembly, overall reaction activity and for background activity attributable to 5 S contamination. Those samples deemed useful were one to two years old and had activities ranging from 65 to 80 U/mg after background correction. Reconstitution of TC complexes was carried out at 4°C overnight with subunits in a 1:6:12 ratio. TC complexes were separated from free subunits and contaminating proteins by gel filtration chromatography on a Shimadzu HPLC with a TSK 5000SW column. This procedure allowed visualization of multiple TC forms, i.e. 26 S, 18 S, as well as uncomplexed proteins. Fractions were collected from the gel filtration eluate and assayed for TC activity. Those
fractions showing significant TC activity were pooled and were considered TC complex for further assays. The TC activity was normalized to biotin content as determined by a modified Rylatt assay to compensate for potential variation in complex assembly. Figure 26 shows an HPLC profile of the complexes formed with 1.3 S M90T. Each of the 1.3 S proteins used in these studies reconstituted well, but the relative amounts of 26 S and 18 S TC forms varied with each reconstitution. Standardization to biotin content compensates for this variation by expressing activity per functional active site. Authentic TC complexes purified from *P. shermanii* and handled in all assays along with reconstituted complexes were the generous gift of Dr. B. C. Shenoy. Table III shows activities measured with these complexes. Each mutation of methionine 88 reduced TC activity by 77-87% while the same substitution at methionine 90 decreased activity by 0-61%. Clearly, the conserved methionine at position 88 makes a larger contribution to the overall activity of transcarboxylase than the equally conserved methionine at position 90. Threonine had the least effect at both positions while leucine had the greatest effect.

**Carboxylation, Halflife and Partial Reactions of Complexes**

To determine the effect of these mutations on partial reaction activity as well as the halflife and thermodynamic stability of the carboxybiotin bond, it was necessary to generate carboxylated complexes with a radioactive label incorporated in the carboxyl group. Transcarboxylase complexes, assembled and assayed for activity as described above, were carboxylated with [4-14C] oxalacetate to produce 14C-carboxybiotinyl TC complexes as in Methods.
Figure 26. HPLC gel filtration separation of TC complexes

Reconstitution of TC was carried out with authentic 5 S, purified, recombinant 12 S and purified 1.3 S M90T. Assembled complexes were separated from free subunits as described in Methods. The peak at 11.717 min. represents 26 S TC, the shoulder at 17.7 min. contained smaller TC forms while overlapping peaks identified as 23.15 min. contained free subunits.
Table III

Transcarboxylase overall reaction activity of 1.3 S subunit mutants in purified, assembled TC complexes

<table>
<thead>
<tr>
<th>TC - 1.3 S subunit</th>
<th>TC activity (U/nmole biotin)</th>
<th>TC activity (% WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC - <em>P. s.</em> a</td>
<td>140</td>
<td>203</td>
</tr>
<tr>
<td>TC - WT</td>
<td>69</td>
<td>100</td>
</tr>
<tr>
<td>TC - I94L</td>
<td>63</td>
<td>91</td>
</tr>
<tr>
<td>TC - M88T</td>
<td>16</td>
<td>23</td>
</tr>
<tr>
<td>TC - M90T</td>
<td>71</td>
<td>103</td>
</tr>
<tr>
<td>TC - M88C</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>TC - M90C</td>
<td>51</td>
<td>74</td>
</tr>
<tr>
<td>TC - M88L</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>TC - M90L</td>
<td>27</td>
<td>39</td>
</tr>
</tbody>
</table>

a - authentic transcarboxylase complexes isolated from *P. shermanii* (from Dr. Shenoy)
The partial reactions were assayed to determine the ability of TC complexes assembled from wild type or mutant 1.3 S subunits to catalyze the transfer of the carboxyl group from biotin to substrate in either the forward or reverse direction. These reactions provide a method by which to dissect the overall activity and assess the effect of mutation on individual components of the reaction mechanism. Aliquots of individual $^{14}$C-carboxybiotinyl TC complexes were incubated with either pyruvate, for the forward partial reaction, or propionyl-CoA, for the reverse partial reaction. These substrates were used at two concentrations - one approximately equal to the $K_M$ and the other saturating. As shown in Table IV, the M88 mutants showed a greater reduction in activity in both reactions and at both concentrations than the corresponding mutations at methionine 90. In general, the higher concentrations of substrate improved the transfer of carboxyl. The $V_{\text{max}}$ of the forward partial reaction was affected by all of the M88 mutants while in the reverse reaction, only M88T and M88C affected the $V_{\text{max}}$. The activities of the mutants in the reverse partial reaction basically followed the same pattern as they did in the overall reaction. Each mutation of methionine 88 impaired enzyme activity while the corresponding mutations of methionine 90 had little effect on either of the partial transcarboxylation reactions. This inhibition could be due to a direct catalytic role of methionine 88 in transcarboxylation or a structural perturbation caused by the 1.3 S mutations.

The halflives of the carboxybiotinyl TC complexes were determined to test the hypothesis that the inhibition of activity was due to stabilization of the carboxybiotin bond by the alteration of its chemical environment caused by mutation of the conserved methionines.
Table IV

Transfer reaction activity\(^a\) of 1.3 S mutants in TC complexes

<table>
<thead>
<tr>
<th>WT) TC - 1.3 S subunit</th>
<th>Transfer reaction activity with the indicated substrate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25 mM pyruvate</td>
</tr>
<tr>
<td>TC - P. s. b</td>
<td>117</td>
</tr>
<tr>
<td>TC - WT</td>
<td>100</td>
</tr>
<tr>
<td>TC - I94L</td>
<td>90</td>
</tr>
<tr>
<td>TC - M88T</td>
<td>58</td>
</tr>
<tr>
<td>TC - M90T</td>
<td>98</td>
</tr>
<tr>
<td>TC - M88C</td>
<td>65</td>
</tr>
<tr>
<td>TC - M90C</td>
<td>133</td>
</tr>
<tr>
<td>TC - M88L</td>
<td>62</td>
</tr>
<tr>
<td>TC - M90L</td>
<td>98</td>
</tr>
</tbody>
</table>

\(^a\) Transfer reaction activity is a measure of activity in the transcarboxylase partial reactions. Transfer activity with propionyl-CoA measures activity in the TC reaction 1 in its reverse direction while transfer activity with pyruvate as the substrate measures activity in TC reaction 2 in its forward direction. The reactions assayed here are the reverse of the reactions measured by the partial reactions in solution.

\(^b\) authentic transcarboxylase complexes isolated from \textit{P. shermanii} (from Dr. Shenoy)
To determine the half-life of the carboxybiotin intermediate form of TC complexes, \(^{14}\text{C}-\text{carboxybiotinyl TC}\) was quantitated during time courses at 0, 15 and 25 °C in the absence of substrates. The loss of label from the TC complexes in the absence of substrate is a simple first order chemical reaction. The rate of this reaction will be altered by anything which affects the stability of the carboxybiotin bond. Therefore, determination of the rate of spontaneous decarboxylation provides an assay for the effect of our mutations on the activity of the biotin as a function of the stability of the carboxybiotin bond.

The time course of \(^{14}\text{C}\) loss for each TC complex fit the single exponential decay equation; all of the curves fit to the data by NFit (103) had R values greater than 0.99. These R values, or correlation coefficients, are a measure of the quality with which the curve fits the distribution of the experimental points and have a maximum possible value of 1.00. The graphs from the \(P.\ shermanii\) TC half-life determinations shown in Figure 27 are representative of the curves determined for each of the complexes. Each point represents a single determination as described in Methods. The curves overlaid on these data points are the theoretical fits. The rate constant (k) for each complex at each temperature was calculated and transformed to determine the half-life \(t_{1/2}\). These values are presented in Tables V and VI and Figure 28 and the equations used are shown in Methods. The rate of decay of the carboxybiotin bond of each of the mutants was significantly altered under most conditions assayed. The effect of these mutations on the stability of the carboxybiotin bond is evident as altered half-life. The halflives for TC-M90T and TC-M90C at 15 and 0°C were the only rates not significantly different from wildtype. As with overall TC activity, the mutations of methionine 88 demonstrated more dramatic alteration of
Figure 27. Carboxybiotin halflife determination

Radioactivities measured during the time courses of spontaneous
decarboxylation were plotted and fit to the single exponential decay
equation with the NFit software. The plots shown here are of authentic
TC from \textit{P. shermanii} at 0\degree C (A), 15\degree C (B) and 25\degree C (C) and are
representative of comparable plots generated for each of the mutant
complexes.
TC-P.s. half-life at 15 C

TC-P.s. half-life at 25 C
Table V

Rate of spontaneous decarboxylation of carboxylated TC complexes involving 1.3S mutant subunits

\( k \) (the rate of spontaneous decarboxylation) as calculated by NFit from \(^{14}\text{C}\)-
carboxylated TC complex decay timepoints

<table>
<thead>
<tr>
<th>TC - 1.3 S subunit</th>
<th>( 25^\circ\text{C} )</th>
<th>( 15^\circ\text{C} )</th>
<th>( 0^\circ\text{C} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC - P. s. (^b)</td>
<td>0.10 +/- 0.01</td>
<td>0.03 +/- 2.9E-03</td>
<td>2.4E-03 +/- 1.7E-04</td>
</tr>
<tr>
<td>TC - WT</td>
<td>0.10 +/- 0.01</td>
<td>0.03 +/- 3.9E-03</td>
<td>2.8E-03 +/- 8.4E-05</td>
</tr>
<tr>
<td>TC - I94L</td>
<td>0.10 +/- 7.8E-03</td>
<td>0.03 +/- 2.5E-03</td>
<td>2.6E-03 +/- 1.7E-04</td>
</tr>
<tr>
<td>TC - M88T</td>
<td>0.06 +/- 4.6E-03</td>
<td>0.02 +/- 2.1E-03</td>
<td>1.8E-03 +/- 2.1E-04</td>
</tr>
<tr>
<td>TC - M90T</td>
<td>0.07 +/- 5.9E-03</td>
<td>0.03 +/- 3.9E-03</td>
<td>2.7E-03 +/- 2.8E-04</td>
</tr>
<tr>
<td>TC - M88C</td>
<td>0.08 +/- 0.01</td>
<td>0.02 +/- 2.0E-03</td>
<td>1.7E-03 +/- 8.9E-05</td>
</tr>
<tr>
<td>TC - M90C</td>
<td>0.07 +/- 0.01</td>
<td>0.03 +/- 4.2E-03</td>
<td>2.7E-03 +/- 2.4E-04</td>
</tr>
<tr>
<td>TC - M88L</td>
<td>0.08 +/- 5.6E-03</td>
<td>0.02 +/- 1.2E-03</td>
<td>2.1E-03 +/- 1.4E-04</td>
</tr>
<tr>
<td>TC - M90L</td>
<td>0.09 +/- 2.4E-03</td>
<td>0.03 +/- 1.2E-03</td>
<td>3.4E-03 +/- 9.4E-04</td>
</tr>
</tbody>
</table>

\( a \) - the least significant measured value had four significant figures (a precision of
+/- 0.0001\% thus all calculations are rounded to this level)

\( b \) - authentic transcarboxylase complexes isolated from \textit{P. shermanii} (from Dr. Shenoy)
Table VI

Half-life and apparent activation energy values calculated from spontaneous decarboxylation of $[^{14}C]$-carboxylated TC complexes with 1.3 S mutants

<table>
<thead>
<tr>
<th>TC - 1.3 S subunit</th>
<th>$T_{1/2}$ (min$^{-1}$)</th>
<th>app. Ea (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>15°C</td>
</tr>
<tr>
<td>TC - P. s. a</td>
<td>6.85</td>
<td>21.8</td>
</tr>
<tr>
<td>TC - WT</td>
<td>6.7</td>
<td>25.3</td>
</tr>
<tr>
<td>TC - I94L</td>
<td>7.17</td>
<td>24.5</td>
</tr>
<tr>
<td>TC - M88T</td>
<td>12.5</td>
<td>32.2</td>
</tr>
<tr>
<td>TC - M90T</td>
<td>10.3</td>
<td>24.5</td>
</tr>
<tr>
<td>TC - M88C</td>
<td>8.95</td>
<td>37.3</td>
</tr>
<tr>
<td>TC - M90C</td>
<td>9.33</td>
<td>24.0</td>
</tr>
<tr>
<td>TC - M88L</td>
<td>8.75</td>
<td>31.1</td>
</tr>
<tr>
<td>TC - M90L</td>
<td>8.00</td>
<td>31.1</td>
</tr>
</tbody>
</table>

a - authentic transcarboxylase complexes isolated from *P. shermanii* (from Dr. Shenoy)
Figure 28. Halflives of carboxybiotin in TC complexes with 1.3 S mutants

Graphical representation of the halflife data presented in Table VII.
carboxybiotin stability than the corresponding mutations at 90. All three substitutions of M88 increased the halflife of the carboxybiotin significantly at all temperatures tested. The identical substitutions for M90 showed a temperature dependent response. At 25°C the halflives were significantly increased while at other temperatures the halflives were not significantly different from wildtype. This would appear to explain the decreased activity of the M88 mutants by stabilization of the carboxybiotin intermediate causing inhibition of carboxyl transfer. This conclusion clearly does not apply to the M90 mutants. These observations led to questions of how the M88 mutations affect this stabilization of the carboxybiotin bond, what the role is of the conserved M90 if these mutations affect overall activity but not partial reactivity or carboxybiotin stability. The apparent temperature dependence noted with the M90 mutants led to analysis of the rate of spontaneous decarboxylation relative to temperature according to the principles described by Arrhenius (104).

**Halflife and Arrhenius Analysis**

To determine the effect of this series of mutations on the activation energy of the carboxybiotin bond, the rate constants of carboxybiotin decay were plotted against the inverse of the absolute temperature as described by Arrhenius (104). The Arrhenius equation defines the relationship between the rate constant, $k$, and the temperature as a function of activation energy, $E_a$. Application of the Arrhenius equation in this case allows calculation of an apparent activation energy which is a sum of components affecting the stability of the carboxybiotin bond. Representative Arrhenius plots are shown in Figure 29. Methionine 90
Figure 29. Arrhenius analysis of TC complex carboxylbin stability

This Arrhenius plot includes authentic, wild type and 1.3 S I94L to demonstrate the consistency of the activation energy determinations.
Arrhenius Plot
TC complex carboxybiotin stability

+ P. shermanii  △ wild type  ○ TC-194L
substitutions demonstrated a marked temperature dependence of carboxybiotin half-life which should result in an altered slope in the corresponding Arrhenius plot. The altered slope, indicating a change in activation energy, is clearly visible for TC reconstituted with 1.3 S M90C in Figure 30. Here, the rate of decarboxylation is plotted against inverse absolute temperature for wild type and the paired mutations M88C and M90C. The slope of the M88C and wild type lines are nearly identical while the M90C line intersects both other lines. This altered slope translates to a ten percent (2.7 kcal/mole) decrease in apparent activation energy. All of the linear fits in this figure have consistently high R values attesting to the quality of fit. The data were analyzed by a two-tailed t-test of paired means to determine the statistical significance of the differences in the calculated apparent activation energies. The values for the authentic, wild type and 1.3 I94L were found not to be significantly different from each other while all other mutants were found to be significantly different from wild type (p < 0.05). Chemically, a change of 2 kcal/mole is potentially significant to the rate of catalysis. Figure 31 presents a summary of the activation energy data. While the carboxybiotin intermediates of our methionine 88 mutants were all significantly stabilized, the apparent activation energy of the carboxybiotin bond was not uniformly changed. In contrast, the identical substitutions for methionine 90 had moderate effects on half-life but were temperature dependent and showed significantly decreased apparent activation energies relative to 1.3 S WT. Analysis of activation energy thus provides a probe for the effect of M90 mutations and a basis for consideration of the function of this conserved residue.
Figure 30. Arrhenius analysis of TC complex carboxybiotin stability

This Arrhenius plot includes wild type, 1.3 S M88C and 1.3 S M90C to demonstrate the consistency of the activation energy determinations and the clear alteration of slope found with the M90C mutation.
Figure 31. Summary of apparent activation energy values

Graphical representation of Arrhenius plot slopes. The error bars represent calculated standard deviations. The activation energies calculated for all of the mutant complexes are statistically different from wild type ($p < 0.05$) as calculated by the two-tailed $t$-test of paired means with Excel 4.0 software. The activation energy of wild type is shown alone because the same statistical analysis showed the authentic and 1.3 I94L not to be significantly different from wild type.
Apparent Activation Energy
TC complexes carboxybiotin bond

![Bar chart showing apparent activation energy (Ea) in kcal/mole for various TC complexes. The X-axis labels are TC-WT, TC-88L, TC-88C, TC-M88T, TC-M80L, TC-M90C, TC-M80T. The Y-axis represents energy in kcal/mole, ranging from 20 to 25.](chart.png)
DISCUSSION

The reaction mechanism of transcarboxylase involves two partial reactions as well as potential conformational intermediates as the biotin makes appropriate contacts at two independent active sites. Conservation of the two methionine residues symmetrically arranged around the active biocytin has been the subject of extensive study.

Kondo et al (105) studied the ability of the E. coli acetyl-CoA carboxylase biotin carboxylase subunit to carboxylate biotin, biocytin and several synthetic peptides with sequences analogous to the biotinyl hexapeptide sequence of its biotin carboxyl carrier protein (BCCP). Their conclusion from these peptide studies was that the conserved methionine residues provide a hydrophobic microenvironment whose unique characteristics are contributed by what they term a sulfide cluster. One of the peptides studied in their report represented the hexapeptide with both methionines replaced by alanine. This peptide was recognized by biotin carboxylase equivalently to the peptide with both methionines intact but the $V_{\text{max}}$ of its carboxylation was reduced by 75%. This study thus demonstrated a role for the methionine residues in the interaction of the biotinyl subunit with the biotin carboxylase subunit. While studying the substrate function of these BCCP peptide analogs, they did not address the stability of the carboxybiotin bond in these peptide substrates. Conclusions from this study are thus limited to identification of a role for one or both of the methionine residues in formation of a hydrophobic environment which promotes interaction at one of the enzyme’s active sites.
The experiments reported here were designed to allow discrimination between these two conserved methionine residues and to study the role each residue plays in both of the reactions the 1.3 S subunit participates in. By analyzing the spontaneous, non-enzymatic decarboxylation of the carboxybiotinyl form of our mutants, we were able to derive a thermodynamic analysis of the contribution of each methionine to the activation energy of the carboxybiotin bond. While this analysis is of a non-enzymatic process, the thermodynamic parameters derived are characteristic of the mutants and are useful in explaining the enzymatic behavior of the mutants.

This is the first case of symmetrically matched mutations which could be analyzed for their specific thermodynamic and kinetic contributions to the reaction mechanism. While symmetric in the primary sequence of 1.3 S and the conserved biotinyl tetrapeptide, these methionines which flank the biocytin clearly make distinct contributions to TC activity. For the purpose of discussion, the results of these studies and some earlier studies are summarized in Table VII.

For all of the mutants tested, modification of M88 had a more dramatic effect on overall reaction and both partial transcarboxylation reactions than the identical substitutions for M90. The forward and reverse partial reactions both follow the same pattern of activity loss as overall reaction activity. These mutations do not discriminate between the direction of the reaction catalyzed.

Thermodynamically, the apparent activation energy of the carboxybiotin bond reflected a greater contribution from methionine 90. Mutations at methionine 88 uniformly increased the half-life of the carboxybiotin bond without significantly altering its apparent activation energy. It would appear then, that methionine 88 plays its primary role in the movement or positioning of the biotin
Table VII

Summary of 1.3 S mutant studies

<table>
<thead>
<tr>
<th>1.3 S mutant activity (%WT)</th>
<th>12 S activity (%WT)</th>
<th>5 S activity (%WT)</th>
<th>$K_M^{(H_850)}$ (µM)</th>
<th>$K_M^{(A_510)}$ (µM)</th>
<th>$t_{1/2}$</th>
<th>$E_a$ (kcal/mol)</th>
<th>Conformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M88L</td>
<td>20</td>
<td>70</td>
<td>70</td>
<td>NC</td>
<td>NC</td>
<td>↑↑↑↑</td>
<td>-</td>
</tr>
<tr>
<td>M90L</td>
<td>80</td>
<td>95</td>
<td>100</td>
<td>NC</td>
<td>NC</td>
<td>↑↑↓</td>
<td>↓</td>
</tr>
<tr>
<td>M88C</td>
<td>10</td>
<td>50</td>
<td>40</td>
<td>NT</td>
<td>NT</td>
<td>↑↑↑</td>
<td>~↓</td>
</tr>
<tr>
<td>M90C</td>
<td>70</td>
<td>100</td>
<td>80</td>
<td>NT</td>
<td>NT</td>
<td>↑--</td>
<td>↓</td>
</tr>
<tr>
<td>M88T</td>
<td>20</td>
<td>60</td>
<td>40</td>
<td>NT</td>
<td>NT</td>
<td>↑↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>M90T</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>NT</td>
<td>NT</td>
<td>↑--</td>
<td>↓</td>
</tr>
</tbody>
</table>

NC = no change from wild type 1.3 S
NT = not tested
at its active sites without directly affecting catalysis in other than a physical sense. In contrast, mutations of methionine 90 altered the activation energy of the carboxybiotin. This direct energetic affect suggests that methionine 90 must play a role in activating the biotin or carboxybiotin for catalysis.

Correlation of these thermodynamic changes with the kinetic affects seen in the activity assays of these mutants is more difficult. In general terms, the mutations of methionine 88 all resulted in significant loss of activity in each reaction assayed. Qualitatively, the relative increase in stability of the carboxybiotin intermediate correlated with the relative decrease in activity. This altered activity is not a thermodynamic affect since changes in the apparent activation energy did not fit this correlation. In the case of the mutations of methionine 90, decreased apparent activation energy should correlate with increased reactivity. The activity data from these mutants do not fit with this prediction. Partial reaction activities of the M90 mutants were not significantly altered from wild type but overall reaction activity was decreased in 1.3 M90C and 1.3 M90L. In these cases, the decreased activation energy of the carboxybiotin intermediate must be offset by other affects of the mutations.

The side chain of methionine displays unique characteristics which have been hypothesized (106) to provide extraordinary plasticity in surface interactions and a polarizable sulfur moiety to methionine containing polypeptides. Thus, in the conserved tetrapeptide of the 1.3 S subunit, the methionines flanking the biocytin provide an environment in which the sidechains may expose the biocytin and provide little or no steric hindrance to interaction between biocytin and substrate binding sites. Alternatively, the flexibility of these sidechains may be exploited in providing a framework for appropriate interaction at the two different
active sites. From the data presented here, we conclude that M88, by virtue of
this uniquely flexible sidechain, is involved in the physical interactions of biotin
with the two substrate binding sites and M90 interacts directly with the biotin,
possibly providing its polarizable sulfur moiety, to affect the catalysis of carboxyl
transfer.

The sequence similarities discussed above were recently extended by Toh
et al (92) to include the sequences of E2 lipoym subunits and various species of H
lipoym protein sequences. The unifying feature in all of these proteins is the fact
that each is post-translationally modified on a conserved lysine. Most of the
sequence conservation seen in this broader comparison is found in hydrophobic
residues away from the modified lysine, except for a notable tendency for a
hydrophobic residue, ie, I, L, or V, and a glutamic acid four and three residues N
terminal to the lysine respectively. This sequence analysis takes on much more
significance when paired with the NMR determination of the 3-dimensional
structure of an E2 protein by Dardel et al (68,69). Brocklehurst and Perham (67)
used the homology in conjunction with the structure to generalize a folding model
and show how it may be applied to E2 subunits, biotinyl proteins and H proteins.
One critical feature of this model is that in each protein, the modified lysine, and
its surrounding residues, are positioned in a β turn at the end of paired β sheets.
This structure would position the prosthetic group on a flexible arm and suggest
that the side chains of surrounding amino acids would be exposed for interaction
with the prosthetic group or its active sites.

Thus, the combination of techniques used in this study have allowed
discrimination of kinetic and thermodynamic contributions of specific sidechains
to the mechanism of action of transcarboxylase. We conclude that the methionine
at position 90 is involved in catalysis while the methionine at position 88 functions in the conformation of the biotin and its physical interaction at the two active sites. We hope, in the future, to assess the rate limiting step of the transcarboxylase reaction on the premise that methionine 88 may affect the rate of movement of the biotin between active sites.
CHAPTER V
CONTINUING AND FUTURE STUDIES OF THE 1.3 S SUBUNIT OF TRANSCARBOXYLASE

INTRODUCTION

The previous chapter presented an analysis of the effect of tetrapeptide mutations on the 1.3 S subunit’s activity, carboxybiontin stability and activation energy. These data have begun to define the kinetic and thermodynamic effects of alterations in the conserved amino acid side chains near the biocytin. These studies gain significance when discussed in the context of the evolving model of 1.3 S structure and function. It will be of interest to continue these studies with a larger set of mutants.

Continuing mutagenesis of the 1.3 S subunit

Thus far, functional analyses have focused on the N terminal, C terminal and biocytin tetrapeptide regions. As described in reference to the hinge region, there are other residues of interest designated by sequence comparisons which have not been subjected to analysis yet. Beyond those residues which appear significant from sequence homology studies, there are likely to be functionally significant residues which can not be identified as targets by examination of homologous protein sequences. In two sections below, mutations are designed to
extend our most successful target, the conserved biotinyl tetrapeptide, and to extend mutagenic analysis throughout the 1.3 S subunit.
MATERIALS AND METHODS

The following oligonucleotides were used in construction of mutant 1.3 S expression vectors by the cassette method described previously. In each case, the mutation is indicated beside the designation of the oligo as representative of the coding or non-coding strand. These oligonucleotides anneal such that the coding and non-coding strands form the double-stranded cassette which replaces the wild type coding region. Indicated to the right of the coding strand oligo is the restriction endonuclease recognition site lost by the mutant and used as a diagnostic in identifying the newly constructed mutation.

The following oligonucleotides anneal to form cassettes with XhoI 5' ends and BstXI 3' ends. These cassettes replace the 1.3 S region from amino acid 85 to residue 90.

M88G-N: TCGAGGCCGGAAG
M88G-C: CCGGCC
A87S-N: TCGAGTGCATGAAG
A87S-C: ATCGAC

The following oligonucleotides anneal to form cassettes with XhoI 5' ends and SacI 3' ends. These cassettes replace the 1.3 S region from amino acid 85 to residue 94.

E86D-N: TCGACGCCATGAAGATGGAGACCGAGCT
E86D-C: CGGTCTCCATCTCTTATGCG
E91D-N: TCGAGGCCATGAACTGCGAGACCGAGCT
E91D-C: CGGTGTCCATCTTCATGCG
E91Q-N: TCGAGGCCATGAAGATGCAGACCGAGCT
E91Q-C: CGGTCTGCATCTTCATGGCC BstX1-
E91L-N: TCGAGGCCATGAAGATGATCACCAGCAGCT
E91L-C: CGGTGATCATCTTCATGGCC BstX1-
M88L/M90L-N: TCGAGGCCCTGAAGCTGGAGACCCGAGCT
M88L/M90L-C: CGGTCTCCAGCTCTCAGGCCC BstX1-
E86D/M88T-N: TCGAGGCCACGAAGATGGAGACCCGAGCT
E86D/M88T-C: CGGTCTCCATCTTCGTGGCC XhoI-
RESULTS

FURTHER MUTAGENESIS

ptac1.3 M88G and A87S

Several additional 1.3 S mutants have either been designed or constructed although not yet fully tested. Mutants ptac1.3 M88G and A87S fall into the latter category and were constructed as previously described with cassettes of complementary synthetic oligonucleotides. Both mutants were identified by restriction digestion and verified by sequencing. Mutant protein accumulation and biotination were assessed relative to wild type 1.3 S by Western and in vivo biotination with $^{14}$C-biotin in culture. The 1.3 S mutants M88G and A87S were constructed and shown to accumulate significant levels of biotinyl protein. These mutants have not been subjected to further analysis.

Biocytin Hexapeptide

The similarities of amino acid sequences among biotinyl carboxyl carrier proteins have been discussed primarily in relation to the conserved tetrapeptide, PAP and G/V repeats. The BLOCKS (v. 6.0) database (107) at the Fred Hutchinson Cancer Research Center identifies the consensus "biotin-requiring enzymes attachment site" sequence as: hydrophobic (LIVM) - x - tetrapeptide - x_{3} - hydrophobic. Sequence alignments (Fig. 32 and(5)) indicate other residues
which demonstrate significant conservation through evolution. In considering the conserved tetrapeptide and its symmetric methionines, it is intriguing to consider the two glutamic acid residues which, in the 1.3 S protein,
Figure 32. Biotin region sequence homology

Comparison of protein sequences from various biotinyl proteins. Most of these sequences are referenced in the text, other sequence references include (45, 67, 92, 108, 109).
<table>
<thead>
<tr>
<th>P.S.</th>
<th>TC 1.3S</th>
<th>V L V L E A M K M E T</th>
</tr>
</thead>
<tbody>
<tr>
<td>K.P.</td>
<td>OADC</td>
<td>L L I L E A M K M E T</td>
</tr>
<tr>
<td>h PCC</td>
<td></td>
<td>I C V I E A M K M Q N</td>
</tr>
<tr>
<td>r PCC</td>
<td></td>
<td>I C V I E A M K M Q N</td>
</tr>
<tr>
<td>E.g.</td>
<td>ACC</td>
<td>L C I V E A M K M H N</td>
</tr>
<tr>
<td>c ACC</td>
<td></td>
<td>F A E I E V M K M V M</td>
</tr>
<tr>
<td>S.g.</td>
<td>PC</td>
<td>V A V L S A M K M E M</td>
</tr>
<tr>
<td>c PC</td>
<td></td>
<td>L V L S A M K M E T</td>
</tr>
<tr>
<td>r PC</td>
<td></td>
<td>L C V L S A M K M E T</td>
</tr>
<tr>
<td>m PC</td>
<td></td>
<td>L C V L S A M K M E T</td>
</tr>
<tr>
<td>s PC</td>
<td></td>
<td>L V L S A M K M E T</td>
</tr>
<tr>
<td>h PC</td>
<td></td>
<td>L C V L S A M K M E T</td>
</tr>
<tr>
<td>S.g.</td>
<td>UC</td>
<td>L L I I E A M K A E M</td>
</tr>
<tr>
<td>S.m.</td>
<td>BCCP</td>
<td>L M I L E A M K M E N</td>
</tr>
<tr>
<td>t BP</td>
<td></td>
<td>V L V L E A M K M E T</td>
</tr>
</tbody>
</table>
occur symmetrically on both sides of the tetrapeptide. Further examination of this "conserved hexapeptide" indicates that the glutamic acid (E86) N terminal of the biotinyl tetrapeptide is found in the analogous position (three residues N terminal to the conserved lysine) in propionyl-CoA carboxylase, acetyl-CoA carboxylase and in nearly all known sequences of lipoyl E2 dehydrogenase subunits and H proteins (67). All of these proteins bind CoA esters. Conversely, the glutamic acid (E91) C terminal of the biotinyl tetrapeptide is found in all known pyruvate carboxylase sequences as well as the biotinyl proteins of tomato and the bacterial enzymes transcarboxylase and oxalacetate decarboxylase. All of these proteins bind keto acids. These differences in association of the conserved residue with the various biotin enzymes are suggestive of a catalytic role for the upstream residue in the keto acid partial reaction. Similarly, the downstream acid may function in the CoA ester partial reaction.

To extend the mutagenic analysis of the 1.3 S subunit to these symmetric glutamic acid residues, a series of mutations was planned to characterize the contribution of each residue to the catalytic function of transcarboxylase and its partial reactions. The mutations considered ranged from conservative, E \( \rightarrow \) D, to less conservative changes which would maintain side chain volume while altering charge, E \( \rightarrow \) Q, or hydrophilicity, E \( \rightarrow \) I. More drastic mutations were not included in this preliminary examination of the glutamic acids.

Two double mutations were included in this set of constructions. One of these mutations was relevant to the conserved glutamic acids; this mutation extends the E86D mutation by including M88T, thus altering two of the three downstream residues under consideration. The other double mutant was an
extension of the tetrapeptide mutants in that it changed both methionines to leucine.

All of these constructions were made as described previously. Potential mutants were screened by digestion with the restriction endonucleases indicated as diagnostic in the design shown in Materials and Methods. Putative mutants were expressed in E. coli strain CSH26 and analyzed for protein accumulation and biotination by Western blotting with anti-TC and anti-biotin antibodies respectively.

All of these mutants accumulated immunoreactive protein at near wild type levels and these proteins were all similarly biotinated. Figure 33 shows a Western analysis using the anti-biotin antibody from Sigma and demonstrates the expression and biotination of these new 1.3 S mutants. The mutations in these constructs were verified by DNA sequencing carried out by Mr. Santos Diaz.

These mutants will be studied in the transcarboxylase partial reactions and in thermodynamic quantizations as described in Chapter IV. These kinetic and thermodynamic analyses should be sufficient for preliminary characterization of the contributions of these conserved glutamic acid residues to each partial reaction and transcarboxylase function. Additionally, the double mutant in which both methionines were changed to leucines should provide a mutant with more extreme catalytic modification to extend the previous characterizations of the tetrapeptide.
Figure 33. Expression and biotination of the new set of 1.3 S mutants

Expression extracts were prepared by our standard method and analyzed by fractionation on 15% SDS-PAGE followed by Western blotting with the anti-biotin antibody. Lanes 1, 4 and 9 show the extracts of the double mutant 1.3 M88LM90L, lane 2 is 1.3 E91I, lane 3 is 1.3 E91Q, lane 5 is ptac1.3, lane 6 is 1.3 E86D, lane 7 is 1.3 E86DM88T, lane 8 is 1.3I94L and lane 10 is molecular weight standards.
Random Mutagenesis

One limitation inherent in the mutagenic studies of structure and function in the 1.3 S protein is the lack of information on the 3-dimensional structure of the subunit. This deficiency forces site-directed mutagenesis to be planned on the basis of evolutionary conservation in the protein sequences of similar proteins. While these designs have led to some interesting results, a complete understanding of functionally significant residues and their contributions cannot be obtained by comparison to other enzymes. There are likely to be residues of importance to protein interactions and catalytic mechanisms unique to transcarboxylase which will not be found in other biotinyl enzymes.

To study residues of functional significance which cannot be identified by sequence homology, a random mutagenesis technique would be useful. There are several methods by which to introduce mutations at random in a DNA segment. For a random mutagenesis approach to be feasible, the most important requirement is a technique for screening which can identify those mutations which have an effect on function in the resulting protein. If an appropriate screening method is not available a sufficiently large population of mutagenic progeny must be analyzed to be representative of all possible mutants.

It has been shown that biotination of expressed 1.3 S proteins can be assayed by incorporation of $^{14}$C-biotin during growth in culture. The only site-directed mutations which affected biotination directly were of the penultimate residue which must be hydrophobic. It was considered likely that other residues
in the 1.3 S sequence are important for biotination which could not be identified by homology but might be detectable by decreased biotination in random mutants.

To identify all 1.3 S residues which affect biotination, a method was developed by which to generate a population of random mutants and screen them for biotination.

Random mutagenesis can be accomplished either by chemical modification of the DNA bases encoding the protein of interest or by incorporation of incorrect bases during synthesis of the coding DNA. The second method may be accomplished either by replication of the gene by an inaccurate polymerase or by polymerization in the presence of a non-standard nucleotide. Taq polymerase is the enzyme commonly used in PCR replication of genes. This polymerase has a known frequency of misincorporation of approximately 1/1000 which is amplified by the presence of manganese. Titration of manganese in a series of polymerase chain reactions would allow designation of the concentration which would generate, on average, one incorrect base incorporated per length of interest. By this technique, the mutagenesis could be adjusted for amplification of segments of varying length, i.e. the entire 1.3 S gene or just the central region. Replication of the 1.3 S gene by taq polymerase in PCR reactions containing manganese would generate an unbiased set of random mutations throughout a defined segment. The alternative to inaccurate base incorporation during amplification is amplification in the presence of inosine. Inosine is a nucleotide, not normally found in DNA, which, through a property referred to as "wobble pairing", can base pair with A, C, or U. As with the manganese approach, titrations are necessary to determine the inosine concentration which will produce the desired frequency of incorporation.
The alternative to mutagenesis by replication is chemical modification of bases in the coding segment of interest. Potential chemical reagents include sodium bisulfite, nitrous acid, formic acid and hydrazine (110). The targets of each chemical are: sodium bisulfite, C; nitrous acid, C, A, G; formic acid, G, A; and hydrazine, C, T. Sodium bisulfite primarily results in C to T transversions while hydrazine can generate either C to T or T to C transversions by breakage of pyrimidine rings. Nitrous acid preferentially causes C to A and A to G transitions by deamination whereas formic acid depurinates G and A, resulting primarily in G to T or C and A to T mutations.

All of these methods require subcloning of the mutated gene segment. After mutagenesis by any of these techniques, the subcloning ligations must be transformed into E. coli and plated normally on selective media. Colonies can then be picked and streaked onto duplicate nitrocellulose filters on min A culture plates containing $^{14}$C-biotin. Overnight growth under these conditions provides membranes with large colonies metabolically labeled with $^{14}$C-biotin. Lysis of the colonies on these membranes results in filters which can be enhanced and subjected to fluorography to determine relative levels of biotin incorporation. Identification of colonies with reduced biotin content would be followed by DNA analysis to identify what mutations resulted in altered biotination. The same analysis may identify colonies with increased biotination. These analyses should identify 1.3 S mutations altering the capacity for biotination, positively or negatively, or the level of accumulation of the protein. This analysis is independent of structural and functional assumptions and, with an adequate sample size, should provide a complete, unbiased determination of residues affecting biotination.
DISCUSSION

The 1.3 S subunit of transcarboxylase has been the subject of intensive study for several reasons. On the most basic level, this protein is part of a complex enzyme and any attempt to understand this enzyme must include the biotinyl subunit. As a component of this multi-subunit enzyme, the 1.3 S protein has numerous functions worthy of study. The 5 S and 12 S subunits of transcarboxylase can not assemble in the absence of this protein and, even if assembled, the enzyme complex is not active unless the 1.3 S subunit is first biotinated. This biotination of the subunit is a post-translational modification which requires recognition and catalysis by another enzyme, holocarboxylase synthetase. Given the presence of the biotin and assembly of the enzyme complex, the 1.3 S subunit then has interesting functions as carboxyl acceptor and donor in the 12 S and 5 S partial reactions respectively and is critical for shuttling the biotin, and thus the carboxyl, between the two active sites and proper positioning at each active site. Beyond the 1.3 S subunit's role as substrate in the partial reactions and its physical role in positioning for catalysis, it also displays intriguing kinetics in a non-enzymatic decarboxylation reaction which is a useful indicator of sidechain contributions in 1.3 S function.

While all of these properties of the 1.3 S subunit of transcarboxylase are interesting and worthy of study in their own right, most of the same properties are characteristic of all biotin-dependent carboxylases giving conclusions from these studies broader significance. The significance of these studies may be further enhanced by the recent observations that the E2 lipoyl subunit common to various
dehydrogenases displays significant sequence homology. The biotinyl and lipoyl protein domains of these disparate carboxylases and dehydrogenases appear to be phylogenetically related and may display a common structure. The commonalities of these disparate proteins further enhance the relevance of structural and functional observations on the 1.3 S protein to general significance in numerous metabolic reactions. Thus continuing studies of 1.3 S residues which function in biotination, physical structure and catalytic function will have broad significance in metabolism and protein science.
<table>
<thead>
<tr>
<th>name</th>
<th>vector</th>
<th>insert</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
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<td>plac12S+1.3S</td>
<td>pUC19</td>
<td>3.2 Kb SphI/BglII fragment with 12 S and 1.3 S</td>
<td>genes (with Vicki Murtif)</td>
</tr>
<tr>
<td>ptac1.3</td>
<td>pKK223-3</td>
<td>1.3 S gene</td>
<td>monomeric - ptac1.3t is dimer</td>
</tr>
<tr>
<td>ptac1.3</td>
<td>pKK223-2</td>
<td>1.3 S gene</td>
<td>1.3 S with trc promoter</td>
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<tr>
<td>plac1.3</td>
<td>pTZ18</td>
<td>1.3 S gene</td>
<td>phagemid in JM109 and CSH26</td>
</tr>
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<td>M13mp18</td>
<td>1.3 S gene</td>
<td>bacteriophage in JM109</td>
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<td>ptac1.3 AP</td>
<td>pKK223-3</td>
<td>1.3 S gene modified to include BamHI site, encodes</td>
<td>1.3 S 1-123-ala-pro</td>
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<tr>
<td>ptac1.3 (&quot;1-132&quot;)</td>
<td>&quot;</td>
<td>1.3 S 1-112 with reverse insertion of 112-125</td>
<td>cassette, encodes 1-112-LWIPDLDETLTLALHSFCFGG</td>
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<tr>
<td>ptac1.3 (85-123AP)</td>
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<td>1.3 S AP truncated at residue 85, encodes M-(85-123-AP)</td>
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<td>pJG200</td>
<td>1.3 S gene</td>
<td>β-galactosidase fusion vector</td>
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<td>pJG1.3 (85-123)</td>
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<td>1.3 S gene residues 85-123 as β-galactosidase fusion</td>
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<td>M13-1.3I94L</td>
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<td>1.3 S gene with I94L mutation to create a SacI restriction endonuclease recognition site at the codon for residue 94</td>
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ptac1.3I94L pKK223-3  1.3 S gene with I94L mutation (see above)
ptac1.3 M90C pKK223-3  1.3 S gene with M90C mutation
ptac1.3 M90T pKK223-3  1.3 S gene with M90T mutation
ptac1.3 M88G pKK223-3  1.3 S gene with M88G mutation
ptac1.3 A87S pKK223-3  1.3 S gene with A87S mutation
ptac1.3 E86D  "  1.3 S gene with E86D mutation (with Santos Diaz)
ptac1.3 E91I  "  1.3 S gene with E91I mutation (with Santos Diaz)
ptac1.3 E91Q  "  1.3 S gene with E91Q mutation (with Santos Diaz)
ptac1.3 E91D  "  1.3 S gene with E91D mutation (with Santos Diaz)
ptac1.3 M88L/M90L  "  1.3 S gene with M88L/M90L mutation (with Santos Diaz and Bhami Shenoy)
ptac1.3 E86D/M88T  "  1.3 S gene with E86D/M88T mutation (with Santos Diaz)


84. Vicki Murtil. 1986. personal communication.


103. ISLAND PRODUCTS, Galveston, Tx 1991. NFIT.


