Biochemical characterization of plant biotin-containing enzymes

Tomás Alberto Diez
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Biochemistry Commons, and the Biophysics Commons

Recommended Citation

Diez, Tomás Alberto, "Biochemical characterization of plant biotin-containing enzymes " (1994). Retrospective Theses and Dissertations. 10692.
https://lib.dr.iastate.edu/rtd/10692
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Biochemical characterization of plant biotin-containing enzymes

Diez, Tomás Alberto, Ph.D.
Iowa State University, 1994
Biochemical characterization of plant biotin-containing enzymes

by

Tomás Alberto Diez

A Thesis Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved:
Signature was redacted for privacy.

In Charge of Major Work
Signature was redacted for privacy.

For the Major Department
Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1994
DEDICATION

This thesis is dedicated to my wife Telvia, and my children Tommy, Luigy, and Alexandra Nicole, who shared with me many sacrifices to see my dream come true. This thesis is also dedicated to the memory of our daughter, Aileen.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GENERAL INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>Rationale and Significance</td>
<td>1</td>
</tr>
<tr>
<td>Biotin</td>
<td>2</td>
</tr>
<tr>
<td>Biotin-Containing Enzymes</td>
<td>4</td>
</tr>
<tr>
<td>Kinetic Mechanism of Biotin-Containing Enzymes</td>
<td>13</td>
</tr>
<tr>
<td>Plant Biotin-Containing Enzymes</td>
<td>15</td>
</tr>
<tr>
<td>Properties of Plant Biotin-Containing Enzymes</td>
<td>16</td>
</tr>
<tr>
<td>Dissertation Organization</td>
<td>17</td>
</tr>
<tr>
<td>References</td>
<td>18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Title</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PURIFICATION AND CHARACTERIZATION OF</strong> 3-METHYLCROTONYL-CoA CARBOXYLASE FROM LEAVES OF <em>Zea mays</em>**</td>
<td>22</td>
</tr>
<tr>
<td>Abstract</td>
<td>22</td>
</tr>
<tr>
<td>Introduction</td>
<td>23</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>24</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>28</td>
</tr>
<tr>
<td>Summary</td>
<td>50</td>
</tr>
<tr>
<td>References</td>
<td>52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Title</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KINETIC ANALYSIS OF THE ACTIVATION OF MAIZE 3-METHYLCROTONYL-CoA CARBOXYLASE BY POTASSIUM IONS</strong>*</td>
<td>56</td>
</tr>
<tr>
<td>Abstract</td>
<td>56</td>
</tr>
<tr>
<td>Introduction</td>
<td>57</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>57</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>58</td>
</tr>
<tr>
<td>References</td>
<td>69</td>
</tr>
</tbody>
</table>

PURIFICATION AND KINETIC STUDIES OF 3-METHYLCROTONYL-CoA FROM LEAVES OF *Glycine max* 70

- Abstract 70
- Introduction 71
- Materials and Methods 72
- Results 74
- Discussion 86
- References 88

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF PROPIONYL-CoA CARBOXYLASE ACTIVITY FROM *Zea mays*: PROPIONYL-CoA CARBOXYLASE ACTIVITY IN EXTRACTS OF PLANTS IS A SIDE-REACTION OF ACETYL-CoA CARBOXYLASE 90

- Abstract 90
- Introduction 91
- Materials and Methods 92
- Results 94
- Discussion 112
- References 114
INTRODUCTION

Rationale and Significance

Biotin, is a heterocyclic organic molecule containing nitrogen and sulphur. This molecule is an essential cofactor for a group of enzymes that catalyze the transfer of a carboxyl group from one substrate (the donor substrate) to another (the acceptor substrate), acting as an intermediate carboxyl-carrier in the reaction. In animals and bacteria, biotin-containing enzymes play important roles in gluconeogenesis, amino acids metabolism, lipogenesis, and energy transduction (1). Any genetic or environmentally induced alteration in the biosynthesis or biochemical use of biotin is fatal in prokaryotic and eukaryotic organisms (2-6). The essential nature of biotin has spurred extensive research on the structure, regulation, and metabolic functions of biotin-containing enzymes. Most of these studies have been undertaken with animals or microorganisms. In stark contrast, knowledge about biotin-containing enzymes of plants is extremely limited. Indeed, acetyl-CoA carboxylase (ACCase) was the only biotin-containing enzyme known in plants until 1990, when Wurtele and Nikolau discovered that plants contain, at least, five other biotin-containing proteins (7). Furthermore, these workers detected 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase activities in extracts from plant tissues (7).

To begin the characterization of these newly discovered plant biotin-containing enzymes, methods for the purification of 3-methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase were devised. The purified enzyme preparations were used to ascertain the structure and catalytic properties of the two enzymes. The studies of 3-methylcrotonyl-CoA carboxylase had the following specific goals:

1. Determination of the structure of 3-methylcrotonyl-CoA carboxylase.
2. Kinetic characterization of 3-methylcrotonyl-CoA carboxylase.
3. Characterization of the activation of 3-methylcrotonyl-CoA carboxylase by monovalent cations.


The studies of propionyl-CoA carboxylase had the following specific goals:

1. Determination of the structure of propionyl-CoA carboxylase.

2. Kinetic characterization of propionyl-CoA carboxylase.

3. Characterization of the nature of propionyl-CoA carboxylase.

In addition, studies were undertaken to determine whether the biotin-containing enzyme, geranoyl-CoA carboxylase is present in plants. These studies lead to the discovery of this enzyme in the plant kingdom. Subsequently, a procedure for the partial purification of this novel biotin-containing enzymes was devised, and the purified enzyme was characterized in terms of its subunit size and catalytic properties.

These studies have provided new insights into the molecular and biochemical properties of plant biotin-containing enzymes.

**Biotin**

The biotin molecule has an imidazolone ring cis-fused to a tetrahydrothiophene ring, which in turn has a valerate side chain. In all known biotin-containing enzymes, biotin is covalently attached to the ε-amino group of a lysine residue forming an amide bond at the active site of the enzyme (Fig. 1A). The biochemical function of biotin is to act as an intermediate carboxyl carrier between the donor and the acceptor substrates of biotin-containing enzymes. As first suggested by Lynen (11), and demonstrated by Guchhait et al. (12), the carboxylation of biotin occurs at N-1 position (Fig. 1B). This carboxylation reaction occurs at one subsite of biotin-containing enzymes, the biotin carboxylase subsite. Primary sequence data of biotin-containing enzymes reveal that this functionally definable
Figure 1. (A) Biotinyl-enzyme. (B) Carboxybiotinyl-enzyme
subsite is physically distinct and conserved among biotin-containing enzymes. The delivery of the carboxyl group from the carboxybiotin intermediate to the acceptor substrate occurs at a distinct, physically separated active site (11, 13-18). Thus, the function of the prosthetic group in biotin-containing enzymes is to act as a physical bridge between physically separate subsites. This function is perfectly suited to biotin since its extended valerate side chain, bound to a lysine residue, can act as a "swinging arm" of about 14 angstrom extended length (the distance from C-5 of biotin to C-2 of lysine) (19). In this regard, biotin-containing enzymes have been compared to the lipoate-containing, α-keto acid dehydrogenases, in which the lipoate prosthetic group transfers the acyl group to different active sites (20).

An important feature of biotin is its noncovalent interaction with avidin, a basic glycoprotein from egg white (21), or with streptavidin, a similar protein from *Streptomyces avidinii* (22). These two proteins have an extremely high affinity for biotin; the dissociation constant of the biotin-avidin (or streptavidin) complex is about $10^{-15}$ M$^{-1}$. This characteristic has been widely exploited as a biochemical tool. In this thesis, the purification of biotin-containing enzymes by affinity chromatography on monomeric avidin (23), and the detection of these enzymes by Western blot analysis with $^{125}$I-streptavidin (24), illustrate the utility of this specific and unusually tight interaction between biotin and avidin or streptavidin.

**Biotin-Containing Enzymes**

In eukaryotic and prokaryotic organisms, biotin-containing enzymes are involved in a variety of biochemical functions including gluconeogenesis, lipogenesis, amino acids metabolism, and energy transduction (1). Specifically, biotin-containing enzymes participate in a number of carboxylation, decarboxylation and transcarboxylation reactions (1). The overall reaction [3] catalyzed by biotin-containing enzymes can be split into two half-reactions, the carboxylation of biotin by the donor substrate (D-CO$_2^-$) [1], and the transfer of
the carboxyl group from the carboxybiotinyl intermediate to the acceptor substrate (A) [2] (25).

\[
\text{D-CO}_2^- + \text{E-Biotin} + \text{Mg.ATP} \rightarrow \text{E-Biotin-CO}_2^- + \text{D} + \text{Mg.ADP} + \text{Pi} \quad [1]
\]
\[
\text{E-Biotin-CO}_2^- + \text{A} \rightarrow \text{E-Biotin} + \text{A-CO}_2^- \quad [2]
\]

Overall reaction: \[ \text{D-CO}_2^- + \text{Mg.ATP} \rightarrow \text{D} + \text{A-CO}_2^- + \text{Mg.ADP} + \text{Pi} \quad [3] \]

These two half-reactions occur at physically distinct active sites, the biotin carboxylase and carboxyltransferase subsites, respectively.

Biotin-containing enzymes have been conveniently divided into three classes, based upon the type of reaction they catalyze (19, 25).

**Class I. Biotin-dependent carboxylases**

All known biotin-dependent carboxylases catalyze the ATP-dependent carboxylation of an acceptor molecule (A), using HCO_3^- as the carboxyl donor molecule. Mg^{2+} is absolutely required for activity since the complex [ATP.Mg]^{2+} is the real substrate. The overall reaction of biotin-dependent carboxylases can be dissected into two half-reactions as shown below:

\[
\text{Enz-Biotin} + \text{HCO}_3^- + [\text{Mg.ATP}]^{2+} \rightarrow \text{Enz-Biotin-CO}_2^- + \text{Mg.ADP} + \text{Pi} \quad [1]
\]
\[
\text{Enz-Biotin-CO}_2^- + \text{A} \rightarrow \text{Enz-Biotin} + \text{A-CO}_2^- \quad [2]
\]
\[
\text{A} + \text{HCO}_3^- + [\text{Mg.ATP}]^{2+} \rightarrow \text{A-CO}_2^- + \text{Mg.ADP} + \text{Pi} \quad [3]
\]

where [3] is the overall reaction.

There are six known biotin-dependent carboxylases (19, 25). Their substrates (A) and final products are shown in Table I. Acetyl-CoA carboxylase is universal in its distribution, being in all species in the biosphere. Pyruvate carboxylase, propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase has been characterized from animal and microbial sources (19). Urea carboxylase is found in yeast and unicellular green algae (25). Geranoyl-
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>A</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA carboxylase</td>
<td>Acetyl-CoA</td>
<td>Malonyl-CoA</td>
</tr>
<tr>
<td>Propionyl-CoA carboxylase</td>
<td>Propionyl-CoA</td>
<td>Methylmalonyl-CoA</td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>Pyruvate</td>
<td>Oxaloacetate</td>
</tr>
<tr>
<td>3-Methylcrotonyl-CoA carboxylase</td>
<td>3-methylcrotonyl-CoA</td>
<td>3-methylglutaconyl-CoA</td>
</tr>
<tr>
<td>Geranoyl-CoA carboxylase</td>
<td>Z-Geranoyl-CoA</td>
<td>Isohexenylglutaryl-CoA</td>
</tr>
<tr>
<td>Urea carboxylase</td>
<td>Urea</td>
<td>N-carboxyurea</td>
</tr>
</tbody>
</table>
CoA carboxylase has been found in a few bacteria including *Pseudomaonas citronellolis*, *P. mendocina*, *P. aeruginosa*, and *Acinetobacter* (1).

**Acetyl-CoA carboxylase** catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA. This reaction is considered to be the first committed step in the biosynthesis of fatty acids. The molecular characteristics of acetyl-CoA carboxylases from prokaryotic organisms differ from those of eukaryotic organisms. Acetyl-CoA carboxylase from *E. coli*, for instance, is a complex of three different functional components: biotin-carboxylase, carboxyltransferase, and biotin carboxyl carrier protein (13, 26-28). The biotin carboxylase component is a dimer of two identical subunits, each of 51 kDa, which catalyzes the carboxylation of biotin (26). The carboxyltransferase component is a tetramer of two different polypeptides of 30 kDa and 35 kDa, and catalyzes the transfer of the carboxyl group from biotin to acetyl-CoA (26). The biotin carboxyl carrier protein is a dimer of two identical polypeptides, each of about 22 kDa (28). In contrast, animal and yeast acetyl-CoA carboxylases have all three functions in a single protein, with molecular weights of about 240,000. Acetyl-CoA carboxylase from animals can undergo a polymerization into a filamentous structure (25). This polymerization is coupled to activation of the enzyme. This is thought to be of physiological significance in regulating fatty acid biosynthesis; in that, citrate, the precursor of acetyl-CoA destined for fatty acid biosynthesis, stimulates the polymerization and thus activation of the enzyme. Conversely, the product of fatty acid biosynthesis, fatty acyl-CoAs, promote the depolymerization of the enzyme. In addition to these allosteric effectors of acetyl-CoA carboxylase, the animal enzyme can be regulated by reversible phosphorylation (29).

An intermediate type of acetyl-CoA carboxylase is found in *Streptomyces erythreus* (12), and in *Turbatrix aceti* (30). The enzyme from these sources is composed of two types of subunits: a biotin-containing subunit which probably contains both the biotin carboxyl
carrier and the biotin carboxylase functions; and a nonbiotin-containing subunit which probably contains the carboxyltransferase function.

**Propionyl-CoA carboxylase** catalyzes the conversion of propionyl-CoA to D-methylmalonyl-CoA. This reaction is involved in the catabolism of branched-chain amino acids, branched-chain fatty acids and odd-numbered fatty acids (25). In animals and bacteria, D-methylmalonyl-CoA is further metabolized to succinyl-CoA by the action of methylmalonyl-CoA racemase and the vitamin B₁₂-dependent enzyme, methylmalonyl-CoA mutase. Propionyl-CoA carboxylase has been isolated from pig heart (31), bovine liver mitochondria (32), rat liver mitochondria (33), and *Mycobacterium smegmatis* (34). All of these propionyl-CoA carboxylase have two kinds of subunits, a biotin-containing subunit, and a nonbiotin-containing subunit. All propionyl-CoA carboxylases appear to have a dodecameric quaternary structure, α₆β₆. The primary sequences of human (35) and rat (36) propionyl-CoA carboxylase have been determined.

**3-Methylcrotonyl-CoA carboxylase** occupies a primary position in the elucidation of the mechanism of action of biotin-containing enzymes (25); 3-methylcrotonyl-CoA carboxylase was the first enzyme to be shown to contain biotin. 3-Methylcrotonyl-CoA carboxylase catalyzes the carboxylation of 3-methylcrotonyl-CoA to produce 3-methylglutaconyl-CoA, a reaction essential for leucine catabolism in animals and bacteria (37). 3-Methylcrotonyl-CoA carboxylase has also been implicated in the mevalonate shunt (38). 3-Methylcrotonyl-CoA carboxylase has been extensively studied from mammalian and bacterial organisms. It has been purified from *P. citronellolis* (39), bovine kidney (40), *Achromobacter* (41), and rat liver (42). The enzyme from these systems is composed of two nonidentical subunits, a biotin-containing subunit, and a nonbiotin-containing subunit. In bacteria, the enzyme has an α₄β₄ quaternary structure (39, 43), whereas in animals the enzyme has an α₆β₆ configuration (40). In bacteria, 3-methylcrotonyl-CoA carboxylase is induced when cells are grown in isovalerate as the sole carbon source (39).
**Pyruvate carboxylase** catalyzes the carboxylation of pyruvate to form oxaloacetate. This reaction has a wide variety of metabolic functions, including gluconeogenesis, lipogenesis and anaplerosis. This enzyme has been isolated from a wide variety of organisms (25). With the exception of the enzymes from *A. niger* and *P. citronellolis*, all of these enzymes are allosterically activated by acetyl-CoA (25). All the pyruvate carboxylases studied so far have a tetrameric quaternary structure. A unique feature of pyruvate carboxylase among biotin-dependent carboxylases is the presence of tightly bound paramagnetic manganese in the enzyme molecule. The removal of the metal ion (carried out only in harsh conditions) leads to the irreversible inactivation of the enzyme (25). Apparently, the role of manganese is to fix the ketoacid substrate in the proper orientation and to promote the abstraction of a methyl proton of pyruvate or the removal of the β-carboxyl group of oxaloacetate (25). All pyruvate carboxylases are inhibited by oxalate, due to the formation of a deadly metal-inhibitor complex (25). Pyruvate carboxylase from Baker's yeast contains zinc instead of manganese (44).

**Geranoyl-CoA carboxylase** has been found only in the bacteria *P. citronellolis*, *P. mendocina*, *P. aeruginosa*, and *Acinetobacter* (10). It catalyzes the carboxylation of Z-geranoyl-CoA to produce isohexenylglutaryl-CoA (39). The enzyme can be selectively induced by growing the cells in geranoate or citronellate as sole carbon source (39). This enzyme is involved in the catabolism of acyclic isoprenoids such as geraniol, farnesol, and citral (45). The enzyme is composed of two nonidentical subunits, a biotin-containing subunit of 75 kDa, and a nonbiotin-containing subunit of 63 kDa. The enzyme has an α₄β₄ configuration (39).

**Urea carboxylase** has been found in certain yeasts and in unicellular algae (25). It catalyzes the carboxylation of urea to form N-carboxyurea. In addition to the ATP and Mg²⁺ requirement, urea carboxylase requires K⁺. The enzyme can be induced by growing the cells in urea, but it is repressed by NH₃. Urea carboxylase has been purified from *Candida utilis*.
(46) and *Saccharomyces cerevisiae* (47). Urea carboxylase from *C. utilis* has a native molecular weight of 600,000. The enzyme from yeast is composed of identical, multifunctional subunits (48).

**Class II. Biotin-dependent decarboxylases**

In anaerobic prokaryotes, biotin-dependent decarboxylases act as energy transducers by coupling the decarboxylation of certain acids to the transport of sodium (1). This process occurs in two steps:

\[
\text{Enz-Biotin + R-CO}_2^- \rightleftharpoons \text{R-H + Enz-Biotin-CO}_2^- \tag{1}
\]

\[
\text{Enz-Biotin-CO}_2^- + H^+ + 2\text{Na}^{+\text{in}} \rightleftharpoons \text{E-Biotin + HCO}_3^- + 2\text{Na}^{+\text{out}} \tag{2}
\]

Overall reaction: \( \text{R-CO}_2^- + H^+ + 2\text{Na}^{+\text{in}} \rightleftharpoons \text{R-H + HCO}_3^- + 2\text{Na}^{+\text{out}} \tag{3} \)

where \( \text{Na}^{+\text{in}} \) and \( \text{Na}^{+\text{out}} \) designate sodium ions inside and outside the cell, respectively. Thus, the energy released from the decarboxylation reaction drives the active transport of Na\(^+\). Obviously, these enzymes do not require ATP. Biotin-dependent decarboxylases share two common features: 1) they are specifically activated by Na\(^+\) ions, and 2) they are membrane-bound proteins (49). The three enzymes that belong to this class are shown in Table II.

**Methylmalonyl-CoA decarboxylase** catalyzes the decarboxylation of methylmalonyl-CoA to produce propionyl-CoA. This reaction occurs in the fermentation of lactate by *Micrococcus lactilyticus* (25). The substrate for the enzyme is the isomer (S)-methylmalonyl-CoA. Malonyl-CoA and succinyl-CoA act as noncompetitive inhibitors of the enzyme (25). Methylmalonyl-CoA decarboxylase is a soluble enzyme with an estimated molecular weight of 275,000-300,000.

**Oxaloacetate decarboxylase** is an inducible enzyme in *Aerotacter aerogenes* which catalyzes the decarboxylation of oxaloacetate to form pyruvate (25). It is exclusively
Table II. Biotin-dependent Decarboxylases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>R-CO$_2^-$</th>
<th>R-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylmalonyl-CoA Decarboxylase</td>
<td>Methylmalonyl-CoA</td>
<td>Propionyl-CoA</td>
</tr>
<tr>
<td>Oxaloacetate Decarboxylasease</td>
<td>Oxaloacetate</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>Glutaconyl-CoA Decarboxylase</td>
<td>Glutaconyl-CoA</td>
<td>Crotonyl-CoA</td>
</tr>
</tbody>
</table>
activated by Na³⁺, since Li⁺, NH₄⁺, Rb⁺, Cs⁺, or K⁺ have no effect on the enzyme activity. Oxaloacetate decarboxylase is a cytoplasmic membrane-bound enzyme.

**Glutaconyl-CoA decarboxylase** catalyzes the decarboxylation of glutaconyl-CoA to produce crotonyl-CoA. This is an intermediate reaction in the fermentation of glutamate to acetate and butyrate (49). Glutaconyl-CoA decarboxylase is composed of three different polypeptides, an α subunit of 140 kDa, β subunit of 60 kDa, and γ subunit of 35 kDa. The α subunit is a hydrophobic polypeptide that binds Na⁺ specifically and is the component involved in the transport of Na⁺. The native molecular weight of the holoenzyme is estimated at 470,000. Thus, the enzyme probably has an α₂β₂γ₂ configuration (49).

**Class III. Biotin-dependent transcarboxylase.**

There is only one known biotin-dependent transcarboxylase, methylmalonyl-CoA:pyruvate transcarboxylase (EC 2.1.3.1), which catalyzes the transfer of the carboxyl group from methylmalonyl-CoA to pyruvate, generating propionyl-CoA and oxaloacetate (25). In *Propionibacteria*, it participates in the fermentation of glucose, glycerol or lactate to propionate. The enzyme is composed of three different subunits: a 12S subunit, a 5S subunit, and a 1.3S-biotin-containing subunit (1). The transcarboxylation reaction is carried out in two partial reactions:

\[
\text{12S subunit} \\
\text{Methylmalonyl-CoA} + 1.3S\text{-Biotin} \rightleftharpoons \text{Propionyl-CoA} + 1.3S\text{-Biotin-CO}_2^- \quad [1]
\]

\[
\text{5S subunit} \\
1.3S\text{-Biotin-CO}_2^- + \text{Pyruvate} \rightleftharpoons \text{Oxaloacetate} + 1.3S\text{-Biotin} \quad [2]
\]

\[
\text{Methylmalonyl-CoA} + \text{Pyruvate} \rightleftharpoons \text{Propionyl-CoA} + \text{Oxaloacetate} \quad [3]
\]

where [3] is the overall reaction. The enzyme only uses (S)-methylmalonyl-CoA as substrate (25). It has been purified from *Propionibacterium shermanii* (50). The holoenzyme is a huge complex composed of 30 polypeptides (1). The central part of the enzyme complex
contains six monomers of 60 kDa (12S subunit); three dimeric 5S subunits, composed of identical monomers of 60 kDa, are located on each face of the 12S subunit hexamer; two identical monomers of 12 kDa (1.3S subunit) interact with each 5S dimer, serving as connectors of the 12S and 5S subunits, in addition to their carboxyl carrier function (1).

**Kinetic Mechanism of Biotin-Containing Enzymes**

Biotin-containing enzymes catalyze the transfer of a carboxyl group from one substrate to another via two half reactions that occur at physically separate active sites, involving three substrates and three products. Thus these enzymes exhibit interesting but complex kinetic mechanisms. Indications that probably all biotin-containing enzymes follow a general Ping-Pong mechanism came from kinetic studies on acetyl-CoA carboxylase and transcarboxylase (25). However, different biotin-containing enzymes follow different Ping-Pong mechanism. It has been demonstrated that some biotin-containing enzymes follow an ordered Bi Bi Uni Uni Ping-Pong mechanism (51, 52), according to the nomenclature of Cleland, whereas other exhibit a random Bi Bi Uni Uni Ping-Pong mechanism (53-55). These two kinetic mechanisms are illustrated in Figure 2, using acetyl-CoA carboxylase as an example. In the first half reaction, bicarbonate reacts with ATP to form carboxyphosphate (56, 57) which reacts with biotin to form the intermediate carboxybiotinyl. After translocation of the intermediate to the second subsite, the carboxybiotinyl group reacts with the final acceptor substrate to form the product. The chemical mechanism of this second half-reaction is not well defined.
Figure 2. (A) Ordered Bi Bi Uni Uni Ping Pong mechanisms with ATP (1) and \( \text{HCO}_3^- \) (2) as the first substrate. (B) Random Bi Bi Uni Uni Ping Pong mechanism.
Plant Biotin-Containing Enzymes

In contrast to the extensive characterization of biotin-containing enzymes from animal and microbial organisms, knowledge of plant biotin-containing enzymes is extremely limited. This is illustrated by the fact that until 1990, acetyl-CoA carboxylase was the only biotin-containing enzyme known in plants (7). However, the suspicion that higher plants contain other biotin-containing enzymes arose in 1985 when Nikolau et al. discovered the existence of four biotinylated polypeptides of 62, 50, 34, and 31 kDa in different plant species by western blot analysis with $^{125}$I-streptavidin (58). The existence of other biotin-containing enzymes in plants was definitely established in 1990 when Wurtele and Nikolau discovered that plant extracts can catalyze 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase and propionyl-CoA carboxylase activities (7). Subsequently, the search for the enzymes responsible for these activities began. Thus, in 1992, Wurtele and Nikolau reported the differential accumulation of biotin-containing enzymes during carrot somatic embryogenesis (59). That study confirmed the existence of 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase, and propionyl-CoA carboxylase activities. In the same year, Baldet et al. reported that green pea mitochondria contain only one biotinylated polypeptide of 76 kDa, which was identified as the biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase (60). One year later, the isolation, purification and partial characterization of this enzyme from somatic embryos of Daucus carota (61), pea leaves and potato tuber (62) and maize leaf (63) were reported. More recently, Song (64), Wang (65), and Weaver (66) cloned and characterized cDNAs from soybean, tomato, and A. thaliana, respectively, coding for the biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase.
Properties of Plant Biotin-Containing Enzymes

To date, acetyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase are the only biotin-containing enzymes that have been purified from plant sources.

Acetyl-CoA carboxylase is the most extensively studied; it catalyzes the first committed step in the biosynthesis of fatty acids. The structure of plant acetyl-CoA carboxylases is unclear since acetyl-CoA carboxylases with biotin-containing subunits, ranging from 21 to 240 kDa, have been reported (67-76). A number of studies have reported the purification of acetyl-CoA carboxylases containing a large, 220 kDa, biotin-containing subunit (74-76). In addition, a recent report indicates that plants may contain a second, multicomponent type of acetyl-CoA carboxylase, as occurs in bacteria (73). This acetyl-CoA carboxylase appears to be composed of three different subunits, 91, 87, and 35 kDa, with the 35 kDa polypeptide containing biotin (73). Thus, it appears that plants may contain two types of acetyl-CoA carboxylases, an eukaryotic multifunctional protein (three functions on one polypeptide) and a prokaryotic multicomponent enzyme (three functions are located on separate subunits). These may occur at different subcellular locations in the plant cell. Perhaps, the existence of different forms of acetyl-CoA carboxylases can be explained in terms of the multiple metabolic processes for which malonyl-CoA is required. Malonyl-CoA is utilized in the biosynthesis of fatty acids (77), very long-chain fatty acids (78), flavonoids (79), stilbenoids (80), anthroquinones (81), malonyl derivatives of D-amino acids (82), malonation of 1-aminocyclopropane carboxylic acids (83), and malonic acid (84).

3-Methylcrotonyl-CoA carboxylase has been recently, and simultaneously purified from carrot somatic embryos (61) and from pea leaves and potato tuber (62) and maize leaves (63). Pea leaf and potato tuber 3-methylcrotonyl-CoA carboxylases are composed of two nonidentical subunits, a biotin-containing subunit of 76 and 74 kDa, and a nonbiotin-containing subunit of 54 and 53 kDa, respectively. Native molecular weights of the enzymes
are 530,000 in pea leaf and 500,000 in potato tuber. Based upon the biotin content of the enzyme, it was suggested that the enzymes have an $A_4B_4$ configuration. Steady-state kinetic analyses suggested that pea leaf 3-methylcrotonyl-CoA carboxylase follows a Ping-Pong mechanism. The purified pea leaf enzyme was inhibited by the final products ADP and Pi, and by 3-hydroxy-3-methylglutaryl-CoA. The enzyme was also inhibited by protein-modifying reagents including N-ethylmaleimide and phenylglyoxal.

Carrot somatic embryo 3-methylcrotonyl-CoA carboxylase is also composed of two nonidentical subunits, a biotin-containing subunit of 78 kDa, and a biotin-free subunit of 65 kDa. In contrast to pea leaf and potato tuber 3-methylcrotonyl-CoA carboxylases, the native molecular weight of this enzyme is about 987,000, suggesting an $\alpha_6\beta_6$ configuration. This enzyme was inhibited by acetoacetyl-CoA and palmitoyl-CoA. All the four 3-methylcrotonyl-CoA carboxylases have a pH optimum around 8.0. The regulation and the biochemical functions of these enzymes is still not clear in plants.

**Dissertation Organization**

This dissertation is composed of a general introduction describing the knowledge of biotin-containing enzymes, five papers (one already published in Arch. Biochem. Biophys.), and a general summary. Chapter 1 presents the isolation, purification, and characterization of 3-methylcrotonyl-CoA carboxylase from maize leaves. Chapter 2 presents the study of the activation of 3-methylcrotonyl-CoA carboxylase by $K^+$. Chapter 3 presents the kinetic mechanism that 3-methylcrotonyl-CoA carboxylase follows. In this later paper, my contribution includes the derivation of the kinetic equations and direct involvement in the kinetic experiments. Chapter 4 explores the nature of propionyl-CoA carboxylase in maize leaves. Finally, Chapter 5 presents the discovery, purification, and partial characterization of a novel biotin-containing enzyme in plants, geranoyl-CoA carboxylase.
References


66. Weaver, L. Manuscript in preparation
PURIFICATION AND CHARACTERIZATION OF 3-METHYLACROTONYL-COENZYME-A CARBOXYLASE FROM LEAVES OF ZEA MAYS

A paper published in Archives of Biochemistry and Biophysics¹
Tomás A. Diez*,² Eve Syrkin Wurtele#, and Basil J. Nikolau*,³

Abstract

3-Methylcrotonyl-CoA carboxylase has been purified to near homogeneity from maize leaves. The resulting preparations of 3-methylcrotonyl-CoA carboxylase have a specific activity of between 200 and 600 nmol·min⁻¹·mg⁻¹ protein, representing an approximately 5,000-fold purification of the enzyme. The purified 3-methylcrotonyl-CoA carboxylase has a molecular weight of 853,000 ± 34,000 and is composed of two types of subunits, a biotin-containing subunit of 80 ± 2 kDa and a nonbiotin-containing subunit of 58.5 ± 1.5 kDa. These data suggest that the enzyme has an a₆b₆ configuration. The optimum pH for activity is 8.0. The kinetic constants for the substrates 3-methylcrotonyl-CoA, ATP and HCO₃⁻ are 11 μM, 20 μM, and 0.8 mM, respectively. Kinetic studies of the 3-methylcrotonyl-CoA carboxylase reaction with variable concentrations of two substrates confirmed that ATP and HCO₃⁻ bind sequentially to the enzyme and that ATP and 3-methylcrotonyl-CoA bind in ping-pong fashion. However, similar analyses indicate that the binding of HCO₃⁻ at the first site is affected by 3-methylcrotonyl-CoA. Kinetic studies of

¹Reprinted with permission from Archives of Biochemistry and Biophysics 1994, 310, 64-75.
²Recipient of a Fulbright-LASPAU Doctoral Scholarship.
³To whom correspondence should be addressed.

*Department of Biochemistry and Biophysics, and #Department of Botany, Iowa State University, Ames, IA 50011
the role of Mg\(^{2+}\) in the 3-methylcrotonyl-CoA carboxylase reaction establish that Mg\(^{-}\)ATP is the substrate for the enzyme, that free ATP is an inhibitor, and that free Mg\(^{2+}\) is an activator. Both Mn\(^{2+}\) and Co\(^{2+}\) can substitute somewhat for Mg\(^{2+}\), but Zn\(^{2+}\) is unable to do so. In addition to carboxylating 3-methylcrotonyl-CoA, the maize carboxylase can carboxylate crotonyl-CoA, but not acetoacetyl-CoA. In fact, acetoacetyl-CoA is a potent, noncompetitive inhibitor, which indicates that the enzyme contains an acetoacetyl-CoA binding site that is independent of the active sites. The monovalent cations K\(^{+}\), Cs\(^{+}\), Rb\(^{+}\), and NH\(_4\)^{+} activated 3-methylcrotonyl-CoA carboxylase activity, with Rb\(^{+}\) being the most potent activator. The inhibition of 3-methylcrotonyl-CoA carboxylase by sulfhydryl and arginyl modifying reagents could be partly alleviated by the substrates ATP and 3-methylcrotonyl-CoA, which suggests that sulfhydryl and arginyl residues may be involved in catalysis.

**Introduction**

Biotin is covalently bound to a set of enzymes that catalyze the transfer of a carboxyl group between two substrates. Biotin functions in these enzymes as an intermediate carrier of the carboxyl group that is being transferred. In contrast to the extensive characterizations of biotin enzymes from animal and microbial sources (reviewed in references 1-5), knowledge of biotin enzymes from the plant kingdom is meager (6,7). The discovery of multiple biotin-containing polypeptides in plant extracts (6) led more recently to the discovery of 3-methylcrotonyl-CoA carboxylase (MCCase\(^{®}\); EC 6.4.1.4) activity in cell free extracts of plants (7-9). This is only the second biotin enzyme to be characterized from this kingdom.
MCCase catalyzes the ATP-dependent carboxylation of 3-methylcrotonyl-CoA to form 3-methylglutaconyl-CoA, a reaction essential for leucine catabolism in animals and microbes (10, 11). The biochemistry of leucine catabolism is less clear in plants, although circumstantial evidence indicates that the pathway may be similar to that in animals and microbes (12). However, in addition, there is a suggestion that leucine can be catabolized in plants by an alternate series of reactions that do not require MCCase (13, 14). To investigate the metabolic function of MCCase in the plant kingdom, and to further characterize the structure and enzymatic properties of this newly discovered biotin enzyme in plants, we undertook its purification and characterization from leaves of maize.

Materials and Methods

Plant material. Maize plants were grown from seed in a greenhouse at 25-30°C with a supplemented daylength of 14 h/day. Leaves were harvested 12-15 days after planting and immediately frozen in liquid N2.

Radioisotopes and chemicals. The radiochemicals NaH\(^{14}\)CO\(_3\) (52.4 mCi/mmol) and Na\(^{125}\)I were purchased from Amersham. The Bradford (15) protein reagent was purchased from Bio-Rad. Q-Sepharose ion exchange resin and Sephacryl S-400 were purchased from Pharmacia. Streptavidin was purchased from Promega. Streptavidin was labeled with carrier-free Na\(^{125}\)I as described previously (16) to a specific radioactivity of 8 x 10\(^7\) cpm/μg of streptavidin. High molecular weight protein standards were purchased from Gibco-BRL. Biochemicals were purchased from Sigma.

Extraction of MCCase. Maize leaves (200-1,000 g) were frozen with liquid N\(_2\) and pulverized in a mortar with a pestle. The resulting powder was homogenized at 4°C in a Waring blender for 1-3 min with 3-5 volumes of 0.1 M Hepes-KOH (pH 7.0), 20 mM 2-mercaptoethanol, 0.1 mg/ml PMSF, 0.1% (v/v) Triton X-100, 1 mM EDTA, and 20% (v/v)
glycerol. The mixture was filtered through several layers of cheesecloth, and the filtrate was immediately centrifuged at 12,200 g (Sorval GS-3 rotor) for 30-40 min. The supernatant (crude extract) was recovered, and the pellet discarded.

Purification of MCCase. Unless otherwise stated, the purification of MCCase was done at 4°C.

Step 1. Polyethylene glycol precipitation. Finely powdered PEG 8000 was slowly added to the crude extract to a final concentration of 4 g PEG/100 ml. The solution was constantly stirred until the PEG was completely dissolved. The mixture was then centrifuged at 12,200 g for 30 min. The supernatant was retained, and more PEG was added and dissolved, to a final concentration of 16 g PEG/100 ml. The precipitate (the 4-16% (w/v) PEG fraction) was collected by centrifugation and resuspended in Buffer A, which was composed of 10 mM Hepes-KOH (pH 7.0), 1 mM EDTA, 20 mM 2-mercaptoethanol, and 20% (v/v) glycerol.

Step 2. Hydrophobic interaction chromatography. The 4-16% (w/v) PEG fraction was applied to a Propyl-N-Agarose column (2.5 cm x 45 cm), previously equilibrated with Buffer A. The column was washed with Buffer A until the A280 of the eluate was less than 0.05. Elution of the enzyme was achieved with a linear gradient of 0 M-0.5 M KCl in Buffer A, at a flow rate of 0.5-1 ml/min. The fractions containing MCCase activity were pooled and used immediately for further purification or frozen in liquid N2.

Step 3. Affinity chromatography on Cibacron-Blue Agarose. The pooled fractions from the hydrophobic column containing MCCase were applied to a column of Cibacron-Blue Agarose (2.5 cm x 10 cm) previously equilibrated with Buffer A. The column was washed with 5 column volumes of Buffer A to remove unbound proteins. Elution of MCCase was achieved with an 800-ml linear gradient of 0 M-1.5 M KCl in Buffer A at a flow rate of 1-1.5 ml/min. Fractions containing MCCase activity were pooled.
Step 4. Ion exchange chromatography on Q-Sepharose. The pooled MCCase preparation obtained from the Cibacron-Blue Agarose column was dialyzed against 4 l of Buffer A for 3-6 h. The dialyzed sample was then passed through a Q-Sepharose column (2.6 cm x 18 cm) that had been previously equilibrated with buffer A. The column was washed with 10 volumes of Buffer A. The enzyme was then eluted by using an 800 ml linear gradient of 0 M-0.75 M KCl in Buffer A. Fractions containing MCCase were pooled and stored at -20°C. Such purified MCCase preparations were used for all kinetic and structural studies.

Enzyme assay. MCCase activity was determined as the rate of incorporation of radioactivity from NaH$_{14}$CO$_3$ into the acid-stable product, 3-methylglutaconyl-CoA, by using the method described by Wurtele and Nikolau (7), with slight variations. The standard assay contained up to 10 nmol/min of the enzyme preparation, in a total volume of 200 µl that contained 100 mM Tricine-KOH (pH 8.0), 1 mM ATP, 2 mM MgCl$_2$, 1 mM DTT, 5 mM NaH$_{14}$CO$_3$ (5 mCi/mmol), and 0.2 mM 3-methylcrotonyl-CoA. After a 10 min incubation at 37°C, the reaction was terminated by the addition of 50 µl of 6 M HCl. A 100 µl portion of the reaction mixture was spotted and dried on a piece of Whatmann 3MM paper, and the radioactivity was determined with a Packard Liquid Scintillation Analyzer. Control assays were without 3-methylcrotonyl-CoA. In the assays for the kinetic characterization of the enzyme, the concentration of the appropriate assay constituent was varied as described in the results, but all other constituents were maintained at the concentrations given here. All assays were done in duplicate, and all kinetic parameters were determined at least three times, and the average (± SE) is presented. All Lineweaver-Burk analyses of the kinetic data were statistically analyzed by carrying out a linear regression analysis of the data points.

Electrophoresis and Western blotting analysis. Protein samples were analyzed by polyacrylamide gel electrophoresis in both denaturing and nondenaturing conditions. Protein
samples were denatured by boiling in the presence of SDS, and proteins were fractionated by SDS-PAGE in slabs (16 cm x 18 cm) composed of 12.5% (w/v) acrylamide/0.1% (w/v) bisacrylamide as described previously (17). After electrophoresis, the gel was silver stained (18), stained with Coomassie Brilliant Blue, or subjected to Western blot analysis (9, 18). Proteins were transferred from gels to nitrocellulose filters by using a Milliblot-SDE System, with buffers consisting of 0.3 M Tris-HCl (pH 10.4) and 20% (v/v) methanol (anode buffer 1); 25 mM Tris-HCl (pH 10.4) and 20% (v/v) methanol (anode buffer 2); and 25 mM Tris-HCl (pH 9.4), 40 mM 6-N-hexanoic acid, and 20% (v/v) methanol (cathode buffer). Transfer was at 450 mA for 35 min. After transfer, the nitrocellulose filter was washed with 10 mM Tris-HCl (pH 7.4) containing 0.9% (w/v) NaCl and 0.05% (v/v) Nonidet NP-40 (TS-NP40 solution). The filter was then placed in 5-10 ml of 5% (w/v) BSA dissolved in TS-NP40 solution and incubated at 37°C for 2 h, or overnight at room temperature. Subsequently, 0.5-1.0 x 10^6 cpm of ^125I-streptavidin was added, and the incubation was continued for 2-6 h. After the incubation with ^125I-streptavidin, unbound streptavidin was removed by washing the filter with TS solution (TS-NP 40 without NP-40) for 10 min and, then, 3 times with TS-NP40 solution for 10 min each. The filter was then wrapped in Saran Wrap™ and exposed for an appropriate time to Kodak X-OMAT G film, at -70°C with intensifying screens. The exposed film was developed as recommended by the manufacturer.

Nondenatured protein samples were subjected to exhaustive electrophoresis in polyacrylamide gel slabs (16 x 18 cm x 1.2 mm) composed of a 3-15% (w/v) linear gradient of acrylamide (19). After at least 44 h of electrophoresis, the gel was stained with Coomassie Brilliant Blue or subjected to Western blot analysis as already described.
Results and Discussion

Purification of MCCase. Before attempting the purification of MCCase from maize leaves, we investigated the long-term stability of the enzyme in extracts prepared with various buffers. We found that MCCase extracted with a 0.1 M Tris-HCl (pH 8.0) was relatively unstable; the enzyme was completely inactive 2-3 days after extraction. Either addition of 1% (w/v) BSA to the enzyme preparation or changes in the pH of the buffer failed to stabilize the enzyme. This problem was overcome by changing the extraction buffer to 0.1 M Hepes-KOH (pH 7.0) and the inclusion of 20% (v/v) glycerol. In addition, all the chromatographic purification procedures were performed in 10 mM Hepes-KOH (pH 7.0) including 20% (v/v) glycerol. In these conditions, MCCase could be stored at -20°C for at least 3 months without any loss in activity (data not shown).

Typical results obtained by the procedure finally adopted for the purification of MCCase are summarized in Table I. MCCase was collected and concentrated from crude extracts by PEG precipitation, with the enzyme precipitating in the fraction between 4% and 16% PEG. This purification step resulted in the apparent activation of the enzyme as judged by the nearly 400% recovery of enzyme activity and a 14-fold increase in specific activity. This apparent activation of MCCase may have been due to the removal of a possible inhibitor(s) of MCCase by the PEG precipitation; alternatively, it may have been due to the underestimation of MCCase activity in the crude extract because of background interference in the assay due to the presence of ribulose-1,5-bisphosphate carboxylase and/or phosphoenolpyruvate carboxylase. In any case, the maize leaf MCCase seems to behave differently from that purified from bovine kidney inasmuch as the latter enzyme was precipitated at 3% PEG (20).
Table I
Purification of MCCase from Maize Leaves

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (mg)</th>
<th>Total Activity (nmol/min)</th>
<th>Recovery (%)</th>
<th>Specific Activity (nmol-min(^{-1}).mg(^{-1}))</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>5,040.0</td>
<td>192</td>
<td>100</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>4-16% PEG</td>
<td>1,295.0</td>
<td>723</td>
<td>377</td>
<td>0.56</td>
<td>14</td>
</tr>
<tr>
<td>Propyl-Agarose</td>
<td>438.0</td>
<td>362</td>
<td>189</td>
<td>0.83</td>
<td>20</td>
</tr>
<tr>
<td>Cibacron Blue</td>
<td>41.8</td>
<td>520</td>
<td>271</td>
<td>12.47</td>
<td>312</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>2.8</td>
<td>566</td>
<td>295</td>
<td>206.23</td>
<td>5,155</td>
</tr>
</tbody>
</table>

*aPurification from 1 kg of plant tissue*
In addition to MCCase, maize leaves contain other biotin-containing proteins, including acetyl-CoA carboxylase, pyruvate carboxylase and propionyl-CoA carboxylase, the last two of which have been relatively recently discovered in plants (7). Although chromatography over propyl-N-agarose gave a small increase in MCCase specific activity, this purification step was retained in the procedure because it separated MCCase from the other biotin enzymes. Subsequent purification by affinity chromatography over Cibacron Blue-agarose and anion exchange chromatography over Q-Sepharose resulted in the overall 5,155-fold purification of MCCase.

The specific activity of MCCase in the final purified preparations ranged between 200 and 600 nmol·min^{-1}·mg^{-1} protein. This is approximately one order of magnitude lower than the specific activity of the MCCases purified from bovine kidneys (20) and Pseudomonas citronellolis (21). This difference in the specific activity of the purified MCCases seems to be due to a lower $k_{cat}$ for the maize enzyme; this lower $k_{cat}$ may be a general property of plant MCCases because, in addition to the maize enzyme, the purified carrot (9) and soybean (unpublished data) MCCases have specific activities of 700 and 600 nmol·min^{-1}·mg^{-1} protein, respectively.

Western blot analysis with $^{125}$I-streptavidin of samples from each purification step demonstrates that purification of the maize MCCase results in the separation and isolation of a single biotin-containing polypeptide of 80 ± 2 kDa (average of four determinations) (Fig. 1A, lanes 1-4). This biotin-containing polypeptide is the biotin subunit of the maize leaf MCCase.

**Subunit Composition of MCCase.** The purified MCCase preparation was analyzed by SDS-PAGE, and the gels were silver-stained to determine the subunit composition of the enzyme (Fig. 1B). The purified preparation contained two predominant polypeptides, the biotinylated polypeptide of 80 kDa, and a biotin-free polypeptide of 58.5 ± 1.5 kDa (average of four determinations). This smaller, biotin-free polypeptide was noticeable in SDS-PAGE
Figure 1. Biotin-containing polypeptides at each step of the purification of MCCase from maize leaves.

A. Aliquots, each containing 0.01 nmol/min of MCCase activity from each fraction of the MCCase purification scheme outlined in the Materials and Methods were subjected to SDS-PAGE and Western analysis with \(^{125}\text{I}\)-streptavidin. A single biotin-containing polypeptide of 80 + 2 kDa is obtained from the purified MCCase. Lane 1, the PEG fraction; lane 2, the propyl-agarose fraction; lane 3, the Cibacron Blue fraction; lane 4, the Q-Sepharose fraction.

B. The purified MCCase preparation obtained from the Q-Sepharose column was subjected to SDS-PAGE and then silver-stained. The purified MCCase contains two polypeptides, one corresponding to the 80-kDa biotin-containing polypeptide, and a second, of 58.5 + 1.5 kDa, which does not contain biotin.
analysis of the fractions containing MCCase activity eluted from the Cibacron Blue-agarose column and the Q-Sepharose column. Elution of the 58.5-kDa biotin-free polypeptide closely followed the elution of MCCase activity and the elution of the 80-kDa biotin-containing polypeptide. These observations led us to believe that the 58.5-kDa polypeptide may be the second biotin-free subunit of MCCase.

To obtain direct evidence that the 58.5-kDa biotin-free polypeptide is indeed in a complex with the 80 kDa biotin-containing polypeptide forming the MCCase holoenzyme, the purified MCCase preparation was subjected to affinity chromatography on immobilized tetrameric avidin. As shown in Fig. 2, the 58.5-kDa biotin-free polypeptide copurifies with the biotin-containing polypeptide during chromatography on immobilized avidin; this finding provides direct evidence that the two polypeptides are physically interacting in the MCCase holoenzyme complex. Thus, the maize leaf MCCase is a heteromeric enzyme composed of two types of subunits, a biotin subunit of 80 kDa and a biotin-free subunit of 58.5 kDa.

**Molecular Weight of the Native MCCase.** Attempts to estimate the molecular weight of the native MCCase by gel filtration chromatography on Sephacryl S-400 were unsuccessful. MCCase activity could not be recovered in the fractions eluted from such a gel filtration column. Western blot analysis of the fractions obtained from the Sephacryl column revealed that the biotin-containing subunit of MCCase eluted over a large number of fractions corresponding to molecular weights of between 280 kDa and 900 kDa. These observations indicated that the functional holoenzyme dissociated in the experimental conditions used during gel filtration chromatography. Similar results have been obtained in our laboratories with MCCase purified from tomato (Xun Wang, personal communication). Further studies are under way to understand this behavior and to determine whether this is a particular characteristic of MCCases from plants. It is of interest that MCCase from *Achromobacter* can be readily dissociated into its constituent subunits by incubation in
Figure 2. Identification of the two subunits of the maize MCCase. The purified maize MCCase (lane 1), was loaded onto an avidin-agarose column (bed volume = 2 ml). The material that did not bind to the avidin-agarose column was collected (lane 2). After extensive washing of the column, biotin-containing proteins bound to the column were eluted with a 2% SDS solution heated to 90°C (lane 3).

A. Equal aliquots of the three fractions were subjected to SDS-PAGE, and the resulting gel was silver stained.

B. Equal aliquots of the three fractions were subjected to SDS-PAGE and Western analysis with $^{125}$I-streptavidin.

Chromatography of the purified maize MCCase on immobilized avidin resulted in the copurification of a 80-kDa biotin-containing polypeptide (lane 3, panel A and B) and a 58.5-kDa biotin-free polypeptide (lane 3, panel A). These two polypeptides are the two subunits of MCCase.
alkaline conditions (22), and a similar dissociation may be occurring during gel filtration of
the maize MCCase.

The molecular weight of MCCase was determined by electrophoresis in
nondenaturing polyacrylamide gradient gels (19). Standard proteins of known molecular
weight and the purified MCCase preparation were subjected to electrophoresis for at least 44
h until migration ceased. Comparison of the migration of the purified MCCase band,
detected by Western analysis with ¹²⁵I-streptavidin, to the migration of the standard proteins
gave an $M_r$ for MCCase of 853,000 ± 34,000 (average of 4 determinations) (Fig. 3). Because
of the lack of a sufficiently large standard protein, the molecular weight of the native
MCCase was obtained by extrapolation of the standard curve. Nevertheless, the four
determinations of the molecular weight of MCCase agreed very closely, with a standard error
of less than 4%. Regardless of the potential systematic error due to the extrapolation of the
standard curve, it is obvious that the maize MCCase has a relative molecular weight
considerably greater than 669,000, which corresponds to the molecular weight of the largest
standard used (bovine thyroglobulin).

Densitometric scans of the silver stained SDS-PAGE gels of the purified MCCase
preparations indicated that the mole stoichiometry of the two subunits is 1:1. Assuming that
the holoenzyme has a molecular weight of about 870 kDa, we suggest that the maize
MCCase may be a dodecamer with an $\alpha_6\beta_6$ configuration. These molecular properties of the
maize MCCase are similar to the MCCase purified from carrot (9). Thus, the plant MCCases
appear to be similar in molecular organization to the bovine kidney MCCase, which also has
an $\alpha_6\beta_6$ subunit organization (20), but these enzymes contrast with the MCCase from
Achromobacter (22) and Pseudomonas citronellolis (21), which have an $\alpha_4\beta_4$ configuration.

**Kinetic Properties of MCCase.**

**pH optimum.** The optimum pH for catalytic activity by the purified MCCase was
determined by varying the pH at which the enzyme was assayed between pH 6.5 to 10.0. To
Figure 3. Estimation of the molecular weight of native MCCase. The purified maize MCCase preparation was subjected to exhaustive electrophoresis in polyacrylamide gels composed of a 3-15% linear gradient of acrylamide (see Materials and Methods). The mobility of MCCase was compared with the mobilities of standard proteins of known molecular weight. The following standard proteins were utilized: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa) and alcohol dehydrogenase (150 kDa). In the determination shown in this figure, the native maize MCCase has an estimated molecular weight of 831,600, and the average of four determinations was 853,000 + 34,000.
avoid effects on MCCase activity caused by differences in the chemical properties of the
different buffers required to control the pH over such a broad range, we used an equimolar
mixture of three buffers (Hepes, Ampso, and Capso) to control the pH of the assay solutions.
The maize leaf MCCase is active over a broad pH range (data not shown). Maximum
activity is attained at pH 8.0, but the enzyme has more than 70% of the maximal activity
between pH 6.8 and pH 9.5. At more alkaline pHs, activity is reduced dramatically; at pH
10.1, the enzyme is only 33% as active as at the optimum pH.

The optimum pH for activity by all MCCases so far examined seem to be near 8.0,
and indeed, this is near the optimum for most biotin enzymes. This may reflect the result of
constraints set by a common feature in the chemical mechanism of catalysis by biotin
enzymes.

**Kinetics of substrate dependence**  The substrates for MCCase are 3-methylcrotonyl-
CoA, HCO3−, and ATP. The kinetic analyses described below were undertaken by observing
the effect on MCCase activity of varying concentrations of one or two substrates at a time
while the other substrate(s) were maintained at a constant, saturating concentration. These
analyses simplified the kinetics by treating MCCase as a "one-substrate" or "two-substrate"
enzyme, respectively.

The response of MCCase activity to increasing 3-methylcrotonyl-CoA, ATP or
HCO3− concentration, in the presence of saturating concentrations of the other substrates, was
in each instance hyperbolic, indicating that the enzyme displayed classical Michaelis-Menten
kinetics. Lineweaer-Burk analyses of these data yielded the following Kms: 3-
methylcrotonyl-CoA, 11 μM; ATP 20 μM; and HCO3−, 0.8 mM. In addition, the Vmax was
calculated to be between 430 and 630 nmol·min−1·mg−1 protein, for each of the substrates
(Table II). Table II also compares the kinetic constants of the maize MCCase with MCCases
isolated from carrot (9), pea (23), *Pseudomonas citronellolis* (24), *Achromobacter* (25), and
bovine kidneys (20). This comparison indicates that the kinetic constants for all these
Table II

Kinetic Constants for 3-Methylcrotonyl-CoA Carboxylases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinetic Constant</th>
<th>Maize(^a)</th>
<th>Carrot(^b)</th>
<th>Pea(^c)</th>
<th>Source of MCCase</th>
<th>Bovine(^f)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(V_{\text{max}})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methylcrotonyl-CoA</td>
<td>630</td>
<td>410</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td></td>
<td>(K_m)</td>
<td>11 ± 3 (\mu)M</td>
<td>42 (\mu)M</td>
<td>50 (\mu)M</td>
<td>43 (\mu)M</td>
<td>12 (\mu)M</td>
</tr>
<tr>
<td>ATP</td>
<td>(V_{\text{max}})</td>
<td>655</td>
<td>121</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td></td>
<td>(K_m)</td>
<td>20 ± 1 (\mu)M</td>
<td>21 (\mu)M</td>
<td>160 (\mu)M</td>
<td>n.r.</td>
<td>83 (\mu)M</td>
</tr>
<tr>
<td>HCO(_3^-)</td>
<td>(V_{\text{max}})</td>
<td>431</td>
<td>420</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td></td>
<td>(K_m)</td>
<td>810 ± 8 (\mu)M</td>
<td>4.0 (\text{mM})</td>
<td>2.2 (\text{mM})</td>
<td>n.r.</td>
<td>2.17 (\text{mM})</td>
</tr>
<tr>
<td>Crotonyl-CoA</td>
<td>(V_{\text{max}})</td>
<td>343</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td></td>
<td>(K_m)</td>
<td>110 ± 12 (\mu)M</td>
<td>n.r.</td>
<td>138 (\mu)M</td>
<td>n.r.</td>
<td>225 (\mu)M</td>
</tr>
</tbody>
</table>

\(^a\) This manuscript
\(^b\) Taken from reference #9
\(^c\) Taken from reference #23
\(^d\) Taken from reference #24
\(^e\) Taken from reference #25
\(^f\) Taken from reference #20

\(\text{Units are nmol/min.mg}\)

n.r. = not reported
MCCases are rather similar. In all examples of this enzyme, the $K_{\text{M}}$s for ATP and 3-methylcrotonyl-CoA are in the range of 10 - 100 µM, whereas the $K_{\text{M}}$s for $\text{HCO}_3^-$ are much higher, ranging 1 - 4 mM.

Kinetic analysis of MCCase as a "two-substrate" enzyme was undertaken to examine the kinetic mechanism of the enzyme. In these studies, we determined the effect on enzyme activity of altering the concentration of two substrates while maintaining a saturating and constant concentration of the third substrate. The double reciprocal plots from these investigations are shown in Fig. 4. The double-reciprocal plot of the initial MCCase activity versus the $\text{NaHCO}_3$ concentration, at three different ATP concentrations, gave a series of linear lines that intercept on the negative x-axis (Fig. 4A). Such intercepting lines are consistent with a sequential kinetic mechanism (23) in which these two substrates bind to the enzyme to form the complex enzyme-ATP-$\text{HCO}_3^-$. When a similar investigation was undertaken to describe the effect on MCCase activity of varying 3-methylcrotonyl-CoA concentration at different concentrations of ATP, the double-reciprocal plot gave a pattern of parallel lines (Fig. 4B). This result is consistent with a ping-pong kinetic mechanism, in which ATP and 3-methylcrotonyl-CoA bind independently of each other and do not form a ternary complex on the enzyme. The results of these two kinetic experiments are in agreement with the kinetic and chemical mechanism that has been previously elucidated for other biotin enzymes (1, 4), namely, a first half-reaction in which the first two substrates, ATP and $\text{HCO}_3^-$, enter the enzyme active site to form the enzyme-ATP-$\text{HCO}_3^-$ complex. Once a chemical reaction takes place to form the enzyme-carboxy biotin intermediate, the first two products, ADP and $\text{P}_i$, leave the enzyme, and the last substrate then enters and reacts with the carboxy biotin intermediate to form the last product.

This mechanism predicts that $\text{HCO}_3^-$ and 3-methylcrotonyl-CoA bind to MCCase in ping-pong fashion, and thus, double-reciprocal plot of MCCase activity versus 3-
Figure 4. Lineweaver-Burk analysis of the dependence of maize MCCase activity on substrate concentrations. The purified maize MCCase preparation was assayed by the procedure described in the Materials and Methods as modified below.
A. The effect on MCCase activity of increasing NaHCO₃ concentration at the indicated constant concentrations of ATP. The 3-methylcrotonyl-CoA concentration was 0.2 mM.
B. The effect on MCCase activity of increasing 3-methylcrotonyl-CoA (MC-CoA) concentration at the indicated constant concentrations of ATP. The NaHCO₃ concentration was 5 mM.
C. The effect on MCCase activity of increasing 3-methylcrotonyl-CoA (MC-CoA) concentration at the indicated constant concentrations of NaHCO₃. The ATP concentration was 1 mM.
D. The effect on MCCase activity of increasing NaHCO₃ concentration at the indicated constant concentrations of 3-methylcrotonyl-CoA (MC-CoA). The ATP concentration was 1 mM.
methylcrotonyl-CoA concentration, at different HCO$_3^-$ concentrations should gave rise to a series of parallel lines. However, when such an analysis was undertaken, a series of nonparallel lines was obtained (Fig. 4C). Indeed, non-parallel lines were also obtained in analyses of the effect of increasing HCO$_3^-$ concentration at constant 3-methylcrotonyl-CoA concentrations (Fig. 4D). The results of these analyses indicate that HCO$_3^-$ and 3-methylcrotonyl-CoA affect each others binding to the enzyme. The discovery of intersecting lines in these 3-methylcrotonyl-CoA-HCO$_3^-$ plots is similar to results obtained for rat liver pyruvate carboxylase (26). These later findings were rationalized by suggesting that the binding of pyruvate affects the binding of HCO$_3^-$.

Similarly, we suggest that for maize MCCase, the binding of 3-methylcrotonyl-CoA affects the binding of HCO$_3^-$.

### Bivalent cation requirement

Biotin enzymes, in general, have a requirement for a bivalent metal ion, usually Mg$^{2+}$, as a cosubstrate (1). Therefore, we investigated the relationship between MCCase activity and the concentrations of ATP and Mg$^{2+}$. To avoid interference due to complexing of Mg$^{2+}$ by assay components other than ATP, such as certain buffers (i.e., Tricine), these studies were conducted with a Hepes-Tris buffer system. Hepes and Tris have a negligible binding constant for Mg$^{2+}$, compared with that of Tricine (27), which was used to buffer the routine assays for MCCase activity.

Figure 5A shows the response of MCCase as the ATP concentration was increased while the Mg$^{2+}$ concentration was maintained constant at 1 mM, 2 mM, or 5 mM. In each instance, the increasing concentration of ATP caused a hyperbolic increase in MCCase activity; peak MCCase activity occurred at equimolar Mg$^{2+}$ and ATP concentrations. Subsequently, as the ATP concentration was increased to values higher than the Mg$^{2+}$ concentration, MCCase activity decreased. We interpret this decrease in activity as being due to the inhibition of the enzyme by free ATP.
Figure 5. The dependence of maize MCCase activity on ATP and Mg$^{2+}$ concentrations. The purified maize MCCase preparation was assayed by the procedure described in the Materials and Methods as modified below.

A. The effect on MCCase activity of increasing ATP concentration at 1 mM (●), 2 mM (◇), and 5 mM (▲) MgCl$_2$. The inset magnifies the low ATP concentration range.

B. The effect on MCCase activity of increasing MgCl$_2$ concentration at 0.05 mM (●), 0.1 mM (◇), and 1 mM (▲) ATP. The inset magnifies the low MgCl$_2$ concentration range.
MCCase showed an absolute requirement for Mg\(^{2+}\). At constant ATP concentrations (0.025 mM, 0.05 mM or 1 mM), increasing the concentration of Mg\(^{2+}\) resulted in the sigmoidal increase of MCCase activity (Fig. 5B). At all ATP concentrations, the maximal MCCase activity occurred at about 2 mM Mg\(^{2+}\), but the absolute level of peak activity increased as the ATP concentration was increased. These results lead us to conclude that the true substrate for MCCase is Mg·ATP, as is true with many other enzymes that require ATP, including probably all biotin enzymes.

One explanation for the initial sigmoidal response of MCCase activity to increasing Mg\(^{2+}\) concentrations when ATP was maintained at constant concentrations (Fig. 5B) is that MCCase was inhibited by the free ATP that prevailed in the assay when the total Mg\(^{2+}\) concentration was less than that of the total ATP concentration. However, this explanation would require that the sigmoidicity in the MCCase response be dependent on the total ATP concentration in the assay and, thus, be most pronounced at the highest ATP concentration. This was not the case. An alternative explanation is that free Mg\(^{2+}\) is an activator of MCCase.

To further investigate the possible activation of MCCase by free Mg\(^{2+}\), MCCase was assayed in the presence of increasing concentrations of ATP and Mg\(^{2+}\) while a constant difference was maintained in their concentrations (Fig. 6). The total ATP concentration was maintained at either 1 mM less than, equal to, or 1 mM greater than, the total Mg\(^{2+}\) concentration in the assay. The concentrations of Mg·ATP, free ATP and free Mg\(^{2+}\) were calculated by using the stability constant for the complex of 70,000 M\(^{-1}\) (28). Figure 6 plots MCCase activity versus the Mg·ATP concentration in the assay. We calculated that, in the assays in which the total ATP concentration was maintained at 1 mM greater than, or equal to, the total Mg\(^{2+}\) concentration, nearly all the Mg\(^{2+}\) in the assay (>98%) is complexed as Mg·ATP. Thus, in these assays, the [Mg·ATP] = [Mg\(^{2+}\)\(_{\text{total}}\)], and the free Mg\(^{2+}\)
Figure 6. The dependence of maize MCCase activity on Mg.ATP concentration. The purified maize MCCase preparation was assayed by the procedure described in the Materials and Methods as modified below.

A. MCCase was assayed in the presence of increasing total ATP and total MgCl₂ concentrations. The relationships between the total ATP and total MgCl₂ concentrations in the assays were:

- \([\text{MgCl}_2] = [\text{ATP}] - 1\text{mM} \) (●)
- \([\text{MgCl}_2] = [\text{ATP}] \) (△)
- \([\text{MgCl}_2] = [\text{ATP}] + 1\text{mM} \) (▲)

MCCase activity is plotted versus the concentration of the Mg.ATP complex in the assay.

B. Hill plot analysis of the data presented in panel A.
concentration in the assay is negligible. In both these conditions, the response of MCCase to increasing Mg-ATP concentration was nearly identical, with a sigmoidal increase in MCCase activity. In the assays containing 1 mM excess total Mg2+, all the ATP is complexed as Mg-ATP, and the assays contain approximately 1 mM free Mg2+. In these conditions, MCCase activity increased hyperbolically as the Mg-ATP concentration was increased. Thus, the effect of introducing free Mg2+ into the assay is to cause a change in the response of MCCase to increasing Mg-ATP concentrations, from sigmoidal to hyperbolic. This alteration in the kinetic behavior of MCCase is further illustrated and quantitated by Hill analysis (29) of the data shown in Fig. 6 (see inset in Fig. 6). When the Mg2+ concentration was increased relative to the ATP concentration, the Hill coefficient decreased from 1.27 to 0.95 and to 0.76. Concurrently, the $K_{0.5V}$ values (i.e., the Mg-ATP concentration at which MCCase activity was half the $V_{max}$) decreased from 2.64 mM to 2.23 mM and to 1.66 mM. These results clearly indicate that free Mg2+ is an allosteric activator of MCCase. Similar activation by free Mg2+ has previously been characterized for pyruvate carboxylase from sheep (30), and acetyl-CoA carboxylase from maize (31); but characterizations that would elucidate activation by free Mg2+ have not been undertaken for other biotin enzymes.

As with many enzymes that require Mg2+ as a cosubstrate, other divalent cations were able to substitute for Mg2+ in supporting MCCase activity. Both Mn2+ and Co2+ were able to substitute for Mg2+; but these cations were able to support MCCase at only 77% and 57%, respectively, of peak MCCase activity obtained with Mg2+ (Fig. 7). Zn2+ was unable to substitute for Mg2+ (data not shown). Interestingly, whereas the initial response of MCCase to increasing Mg2+ and Co2+ concentrations was sigmoidal, the response to increasing Mn2+ was hyperbolic. The cause for this difference may be a higher affinity for Mn2+ at the allosteric site than for Mg2+ or Co2+.

**Effect of substrate analogs.** The purified MCCase was tested to ascertain its specificity in terms of the acyl-CoA that it will carboxylate. 3-Methylcrotonyl-CoA was
Figure 7. The dependence of maize MCCase activity on divalent metal cations. The purified maize MCCase preparation was assayed by the procedure described in the Materials and Methods, but each assay contained the indicated concentrations of MgCl₂, MnCl₂, or CoCl₂.
substituted in the standard assay with a number of different acyl-CoA substrates. n-Butyryl-CoA, n-valeryl-CoA, isobutyryl-CoA, isovaleryl-CoA, n-hexanoyl-CoA and tiglyl-CoA did not serve as substrates for carboxylation. Indeed, all these acyl-CoAs were weak inhibitors of MCCase activity; when each acyl-CoA was presented to MCCase at 0.15 mM (i.e., equimolar with 3-methylcrotonyl-CoA), activity was inhibited by 13%-26%.

Crotonyl-CoA was carboxylated by MCCase, but it was a poorer substrate than 3-methylcrotonyl-CoA; the $K_m$ for crotonyl-CoA was 110 $\mu$M and $V_{max}/K_m = 3.2$ (cf. for 3-methylcrotonyl-CoA: $K_m = 11 \mu M$ and $V_{max}/K_m = 57.3$). These findings for the maize MCCase are comparable to those with the bovine kidney (20), Pseudomonas citronellolis (24) and Achromobacter (25) enzymes; all of the later enzymes could also utilize crotonyl-CoA as an alternative, but poor, substrate.

However, in contrast to the bovine kidney (20) and Achromobacter (25) MCCases, which could carboxylate acetoacetyl-CoA, but as a poor substrate, acetoacetyl-CoA was an inhibitor of the maize MCCase. Acetoacetyl-CoA is also an inhibitor for the carrot MCCase (9). Lineweaver-Burk analyses of the effect of different acetoacetyl-CoA concentrations relative to ATP concentrations (Fig. 8A) and 3-methylcrotonyl-CoA concentrations (Fig. 8B) showed that, for both substrates, acetoacetyl-CoA was a noncompetitive inhibitor of maize MCCase. Increasing the concentration of acetoacetyl-CoA did not affect the $K_m$ for either ATP or 3-methylcrotonyl-CoA, but in each instance it decreased the $V_{max}$. That is, acetoacetyl-CoA did not affect the binding of these two substrates. These results indicate that the maize MCCase has a binding site for acetoacetyl-CoA that is distinct from both the 3-methylcrotonyl-CoA and ATP substrate binding sites.

Secondary plots of the slopes of the lines obtained in the Lineweaver-Burk plots versus the concentration of acetoacetyl-CoA are shown in the insets of Figs. 8A and 8B. From these secondary plots, the inhibition constants ($K_{is}$) were deduced to be $28.2 \pm 1.5 \mu$M
FIG. 8. Lineweaver-Burk analysis of the inhibition of maize MCCase activity by acetoacetyl-CoA. The purified maize MCCase preparation was assayed by the procedure described in the Materials and Methods as modified below.

A. The effect on MCCase activity of increasing ATP concentration in the presence of 0 µM (•), 5 µM (○), 10 µM (♂), and 20 µM (□) acetoacetyl-CoA. The inset shows the secondary plot of the y-intercept of the Lineweaver-Burk plots versus the acetoacetyl-CoA (AA-CoA) concentration. These data indicate that acetoacetyl-CoA is a noncompetitive inhibitor of MCCase relative to ATP, with a Ki of 29.6 µM.

B. The effect on MCCase activity of increasing 3-methylcrotonyl-CoA (MC-CoA) concentration in the presence of 0 µM (■), 3 µM (△), and 6 µM (□) acetoacetyl-CoA. Inset shows the secondary plot of the y-intercept of the Lineweaver-Burk line versus the acetoacetyl-CoA (AA-CoA) concentration. These data indicate that acetoacetyl-CoA is a noncompetitive inhibitor of MCCase relative to 3-methylcrotonyl-CoA, with a Ki of 17.2 µM.
and 11.2 ± 4.9 μM relative to ATP and 3-methylcrotonyl-CoA, respectively (average of three determinations each).

**Effect of monovalent cations.** The addition of monovalent cations to the MCCase assay affected the activity of the enzyme (data not shown). Li⁺ and Na⁺ were slightly inhibitory, each inhibiting MCCase activity by 26%. However, K⁺, Cs⁺, Rb⁺, and NH₄⁺ were activators of MCCase activity. Maximal activation was obtained with Rb⁺, which activated MCCase activity by more than 2-fold. Similar activation by monovalent cations has previously been observed for pyruvate carboxylase from sheep (30) and acetyl-CoA carboxylase from maize (31). The mechanism of activation is not absolutely known, but it has been suggested that monovalent cations activate by the formation of a cation-enzyme complex that stabilizes the transient formation of negative charges during catalysis (32, 33).

**Effect of protein modification reagents.** Reacting MCCase with sulfhydryl and arginyl modifying reagents caused inhibition of enzymatic activity. The arginyl modifying reagent, phenylglyoxal, inhibited MCCase in a time-dependent (Fig. 9A) and concentration-dependent (Fig. 9A, insert) manner. At a concentration of 50 mM phenylglyoxal, 80% of the MCCase activity was inhibited in 20 min. The inclusion of the substrates 3-methylcrotonyl-CoA or ATP partly protected the enzyme from modification by phenylglyoxal; both these substrates were equally effective. Interestingly, when 3-methylcrotonyl-CoA and ATP were added together to the modifying reaction, MCCase was less well protected than when either substrate was added singly.

The sulfhydryl modifying reagent, p-HMBA, was a potent inhibitor of MCCase 10 μM p-HMBA inhibited MCCase by nearly 90% (Fig. 9B). The modification of MCCase by p-HMBA was partly inhibited by either 3-methylcrotonyl-CoA or ATP, and furthermore, the use of both substrates together synergistically protected the enzyme.

With both modifying reagents, it is not clear if the protection afforded by the substrates, is due to direct steric protection of residue(s) or is due to indirect effects of
Figure 9. Covalent modification and inactivation of maize MCCase.

A. The purified maize MCCase preparation was incubated at 37°C for the time indicated with 0 mM (•) or 30 mM (○) phenylglyoxal in the absence of any substrates or with 30 mM phenylglyoxal in the presence of 0.3 mM 3-methylcrotonyl-CoA (○), 1 mM ATP (○), and 0.3 mM 3-methylcrotonyl-CoA plus 1 mM ATP (□). At the times indicated, an aliquot of the reaction was diluted into an MCCase assay, and residual MCCase activity determined. The inset shows the inhibition of MCCase reacted with the indicated concentrations of phenylglyoxal for 20 min at 37°C.

B. The purified maize MCCase preparation was incubated at 37°C with 0 µM (•) or 45 µM (○) p-HMBA in the absence of any substrates or with 45 µM p-HMBA in the presence of 0.3 mM 3-methylcrotonyl-CoA (○), 1 mM ATP (○), and 0.3 mM 3-methylcrotonyl-CoA plus 1 mM ATP (□). At the times indicated, an aliquot of the reaction was diluted into an MCCase assay, and residual MCCase activity determined. The inset shows the inhibition of MCCase reacted with the indicated concentrations of p-HMBA for 10 min at 37°C.
structural changes in the enzyme induced by the binding of the substrates that hide the residue(s) from modification. In either case, it is clear that MCCase requires arginine and cysteine residues for catalytic proficiency.

Summary

MCCase was purified from maize leaves by the use of three chromatographic purification steps, hydrophobic-interaction chromatography, Cibacron Blue affinity chromatography, and anion-exchange chromatography. This purification procedure yields a preparation that is near homogeneity, the result of about 5,000-fold purification. The purified maize MCCase was characterized to ascertain its structural and enzymological properties. The kinetic and structural properties of the maize MCCase is similar to another plant MCCase, purified from the dicotyledonous carrot (9). Therefore, monocotyledonous and dicotyledonous plant species seem to have fairly similar MCCases.

As with MCCases from other kingdoms (20-22), the plant MCCase is a large complex (the native maize enzyme has a molecular weight of about 870,000) and is composed of two types of subunits - a biotin-containing subunit and a biotin-free subunit. The plant MCCase probably is dodecameric in an \( \alpha_6\beta_6 \) quaternary configuration, similar to the bovine kidney enzyme (20), and unlike the MCCases from bacterial sources, which are octameric having an \( \alpha_4\beta_4 \) configuration (21, 22). Therefore, it seems that there may be a clear difference in the quaternary structures of eukaryotic and prokaryotic MCCases. An interesting observation about the maize MCCase that we are currently investigating, is the seeming dissociation of the MCCase under the mild conditions of gel filtration chromatography. This property of the maize MCCase may enable us to ascertain the relative organization of the subunits in the holoenzyme.
The heteromeric nature of animal, bacterial and plant MCCases is similar to that of propionyl-CoA carboxylase (34, 35) and geranyl-CoA carboxylase (21), which are also heteromeric enzymes composed of a biotin subunit and a biotin-free subunit. It is becoming evident, particularly with the molecular cloning and sequencing of genes and cDNAs coding for biotin enzymes (3), that the biotin-containing subunit of propionyl-CoA carboxylase contains the biotin carboxylase and the carboxy-biotin carrier domains while the biotin-free subunit contains the carboxytransferase domain (36). We speculate that this is also the molecular arrangement of MCCase. Indeed, the molecular cloning and sequencing of the biotin subunit of MCCase from soybean, tomato, and Arabidopsis (manuscripts in preparation) confirms this organization for MCCase.

The kinetic characteristics of the MCCase purified from maize show some similarity to MCCases purified from bovine kidney (20), Pseudomonas citronellolis (21), Achromobacter (22), carrot (9), and the partly purified MCCase from pea (23). Some of these characteristics are common to many biotin enzymes, although, the maize MCCase displays characteristics that distinguish it from previously characterized MCCases. The most pronounced difference between plant MCCases and MCCases from other kingdoms is the inhibition of the plant enzyme by acetoacetyl-CoA. Both the bovine kidney (20) and Achromobacter (22) MCCases are able to utilize acetoacetyl-CoA as a poor substrate for carboxylation. In contrast, this compound is a noncompetitive, potent inhibitor of the maize MCCase. The kinetics of acetoacetyl-CoA inhibition of the maize MCCase are noncompetitive with respect to the two substrates, ATP and 3-methylcrotonyl-CoA, which indicates that the enzyme has an acetoacetyl-CoA binding site independent of the active sites that bind ATP and 3-methylcrotonyl-CoA.

The inhibition of MCCase by acetoacetyl-CoA may have physiological significance. Although the metabolic function of MCCase is as yet undefined in plants, extrapolation from animal and bacterial systems would suggest that it has a role in leucine catabolism (10, 11).
and the "mevalonate shunt" (37-39). Leucine catabolism, as elucidated in animals and microbial systems, generates acetyl-CoA and acetoacetate, and the mevalonate shunt diverts mevalonate from isoprenoid biosynthesis to acetoacetate and acetyl-CoA. The acetoacetate can subsequently be activated to acetoacetyl-CoA by its reaction with succinyl-CoA. Should this be the the metabolic function of MCCase in plants, it would be involved in a process that generates acetoacetyl-CoA and acetyl-CoA, which can potentially be used as substrates for respiration, lipogenesis, and in the presence of the glyoxylate pathway, gluconeogenesis (38, 39). These interconversions may be regulated in part by the mediation of MCCase activity, which may be brought about by the feedback inhibition of the enzyme by acetoacetyl-CoA. The physiological significance of this mechanism awaits further investigations.

References


Abbreviations used: DTT, dithiothreitol; p-HMBA, p-hydroxymercuribenzoic acid; MCCase, 3-methylcrotonyl-CoA carboxylase; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.
KINETIC ANALYSIS OF THE ACTIVATION OF MAIZE 3-METHYLACETOACETATE CARBOXYLASE BY POTASSIUM IONS

A paper to be submitted to Biochimica et Biophysica Acta

Tomás A. Diez, Eve S. Wurtele and Basil J. Nikolau

Abstract

Maize leaf 3-methylcrotonyl-CoA carboxylase is activated by monovalent cations, such as K⁺, Rb⁺, Cs⁺, and NH₄⁺, but Li⁺ or Na⁺ have no effect on the activity of the enzyme. (Diez et al., 1994). A kinetic analysis of the effect of K⁺ on MCCase was undertaken to ascertain the specific role of K⁺ in the carboxylation of 3-methylcrotonyl-CoA. These analyses show that K⁺ is a nonessential activator of the enzyme. The kinetic mechanisms by which K⁺ activates 3-methylcrotonyl-CoA carboxylase was studied relative to the three substrates of the enzyme. Relative to bicarbonate, K⁺ is a noncompetitive activator. Furthermore, as ascertained from the effect of the activator on the dissociation constants for the enzyme-bicarbonate complex, K⁺ does not affect the binding of bicarbonate to 3-methylcrotonyl-CoA carboxylase. Relative to ATP and methylcrotonyl-CoA, K⁺ activates the enzyme by a mixed kinetic mechanism. In addition, K⁺ affected the binding of these two substrates to the enzyme. K⁺ positively affected the binding of ATP to the enzyme, but negatively affected the binding of methylcrotonyl-CoA. These results are discussed relative to the mechanism of methylcrotonyl-CoA carboxylation.
Introduction

3-Methylcrotonyl-CoA carboxylase (MCCase) is a biotin-containing enzyme that catalyzes a reaction required in the catabolism of leucine. MCCase has been purified and characterized from bovine kidney (Lau et al., 1980) and certain bacteria (Fall and Hector, 1977; Schiele et al., 1975). This enzyme has recently been identified in plants, and its purification and characterization has been reported from carrot (Chen et al., 1993), maize (Diez et al., 1994), pea and potato (Alban et al., 1993). MCCase purified from maize leaves is composed of two types of subunits, an 80 kDa biotin-containing subunit (α) and a 59 kDa nonbiotin-containing subunit (β) (Diez et al., 1994). The holoenzyme appears to have an α6β6 quaternary arrangement since its native molecular weight is about 850 kDa (Diez et al., 1994). As with other biotin-containing enzymes, it has an absolute requirement for Mg$^{2+}$ due to the fact that the Mg-ATP complex is the enzyme’s real substrate (Diez et al., 1994). In addition, Mg$^{2+}$ is an allosteric activator of MCCase (Diez et al., 1994). In this paper, we report the kinetic characterization of the activation of the purified maize MCCase by monovalent cations.

Materials and Methods

Radioisotopes and Chemicals. NaH$^{14}$CO$_3$ (52.4 mCi/mmol) was purchased from Amersham. Mono-Q-Sepharose ion exchange resin was purchased from Pharmacia. Propyl-Agarose and Cibacron Blue 3GA-Agarose resins and all other biochemicals were purchased from Sigma.

Extraction and Purification of MCCase. MCCase was extracted and purified as described elsewhere (Diez et al., 1994). Aliquots of 2-5 ml of purified enzyme were exhaustively dialyzed against two changes of 5L of 10 mM Hepes-Tris (pH 7.0), 20% (v/v) glycerol, 1 mM EDTA, and 20 mM 2-mercaptoethanol.
**Enzyme assay.** MCCase activity was measured essentially as described by Diez et al. (1994), with the exception that 100 mM Hepes-Tris (pH 8.0) was utilized to buffer the assays. No more than 10 nmol/min of enzyme activity were used in each assay. The effect of the KCl on MCCase was studied by measuring MCCase activity in the absence ($v_0$) and presence ($v$) of increasing amounts of KCl.

**Results and Discussion**

A number of biotin-containing enzymes have been reported to be activated by monovalent cations (Suelter, 1985; Nikolau and Hawke, 1984; Giorgio and Plaut, 1967; Edwards and Keech, 1968). Initial experiments to determine the effect of monovalent cations on purified maize MCCase demonstrated that monovalent cations are nonessential activators of MCCase. Of the cations tested, Rb$^+$ was the most effective activator, followed in decreasing order by NH$_4^+$ and K$^+$ (Diez et al., 1994). To further characterize this activation of MCCase, we undertook a more detailed examination of the kinetic effect of K$^+$ on this enzyme. We chose to use K$^+$ because of the physiological significance of this ion; Rb$^+$ is barely detected in plants and NH$_4^+$ might be toxic at concentrations high enough to promote enzyme activation (Lauchli and Pfluger, 1978).

Figure 1 shows the effect of increasing concentration of KCl on MCCase activity, at saturating concentrations of the substrates of the enzyme. A classical hyperbolic increase in MCCase activity was observed, with maximum activation, of 2-fold, occurring at 50 mM KCl. At higher concentrations of K$^+$, MCCase activity declined. As suggested by Kachmar and Boyer (1953), this deactivation of the enzyme might be due to ionic strength effects which may alter the active conformation of the enzyme molecule. A reciprocal plot of $(v - v_0)$ versus K$^+$ concentration gives a linear line for the data in the range of 0-20 mM KCl (inset of Fig. 1). However, the points subsequently depart from linearity as K$^+$ concentration is
Figure 1. Effect of K⁺ on MCCase activity. The activity of the enzyme was determined, as described in Materials and Methods, in the presence of increasing concentration of K⁺.
increased to cause deactivation of the enzyme. Extrapolation of the linear portion of the line reveals an apparent \( \text{K}_m \) value for \( K^+ \) of 4.6 mM. Most of the enzymes that are activated by \( K^+ \) show an apparent \( \text{K}_m \) close to 10 mM (Suelter, 1970), which is in good agreement with our results.

Although MCCase is a multisubstrate enzyme, it can be studied as a one-substrate system if the concentration of one substrate is varied while the concentrations of the other two substrates are kept at constant, saturating levels (Frieden, 1964). Therefore, to study the mechanism of \( K^+ \) activation, the activity of MCCase was measured at different concentrations of KCl while varying the concentration of each of the three substrates individually, and maintaining the concentrations of the other two substrates at saturating levels.

Such a system can be represented by the general mechanism depicted in Scheme I (Dixon and Webb, 1979). In this system, \( K_s^S \) and \( K_m^S \) are the dissociation constants for the formation of the ES and EAS complexes, respectively; \( K_s^A \) and \( K_m^A \) are the dissociation constants for the formation of the EA and EAS complexes, respectively; and, \( k \) and \( k' \) are the velocity constants for the breakdown of the ES and EAS complexes, respectively.

Assuming equilibrium conditions, this system will obey the following kinetic equation (Dixon and Webb, 1979):

\[
\frac{v}{ke} = \frac{K_s^S \cdot (1 + a/K_m^A)}{(1 + a/K_m^A) + K_s^S \cdot (1 + a/K_m^A) + (1 + k'/k \cdot a/K_m^A)} \cdot \frac{S}{(1 + k'/k \cdot a/K_m^A)}
\]

Double reciprocal plots of \( 1/v \) versus \( 1/S \) will yield linear lines. From these primary plots the slopes and y-intercepts can be determined in the absence (slope\( ^0 \) and y-intercept\( ^0 \),
Scheme I. General mechanism for partial activation.
respectively), and presence of activator (slope<sup>c</sup> and y-intercept<sup>c</sup>, respectively). For each concentration, <i>c</i>, of activator, values of Δslope (= slope<sup>0</sup> - slope<sup>c</sup>) and Δy-intercept (= y-intercept<sup>0</sup> - y-intercept<sup>c</sup>) can be calculated.

A secondary plot of the reciprocals of Δslope and Δy-intercept versus the reciprocal of the activator concentration will yield two linear lines that intercept on the negative x-axis, at a value of (<i>k</i>'<i>k</i>)/<i>K<sub>nA</sub></i>. In addition, the y-intercept of the Δslope line will give <i>kel</i>(<i>K<sub>5S</sub></i> - <i>K<sub>mS</sub></i><i>k</i>'), and the y-intercept of the Δy-intercept line will give <i>kel</i>(1 - <i>k</i>/'<i>k</i>). Since <i>K<sub>s</sub>S</i> and <i>ke</i> can be obtained from Lineweaver-Burk plots in the absence of activator, and <i>K<sub>s</sub>A</i>.<i>K<sub>mS</sub></i> = <i>K<sub>mA</sub></i>.<i>K<sub>s</sub>S</i>, the values of <i>K<sub>s</sub>S</i>, <i>K<sub>mS</sub></i>, <i>K<sub>s</sub>A</i> and <i>K<sub>mA</sub></i> can be obtained.

Figures 2A, 3A and 4A, show the effect of increasing bicarbonate, ATP and methycrotonyl-CoA, respectively, on MCCase activity in the presence of different K<sup>+</sup> concentrations. In all of these analyses, MCCase activity increased hyperbolically as the concentration of the substrate was increased. Lineweaver-Burk analyses of these data (Figs. 2B, 3B, 4B) illustrate the effect of K<sup>+</sup> ions on MCCase, relative to the three substrates. With respect to bicarbonate, K<sup>+</sup> ions affect the <i>V<sub>max</sub></i> of the enzyme but did not affect the <i>K<sub>m</sub></i> for bicarbonate (Fig. 2B). Thus, K<sup>+</sup> acted as a noncompetitive activator relative to bicarbonate. Analysis of these data according to the method discussed above, enabled the determination of the dissociation constants for Scheme I, relative to the substrate, bicarbonate (Fig. 2C). These values (Table I) illustrate that K<sup>+</sup> ions do not affect the binding of bicarbonate to the enzyme, and conversely the binding of K<sup>+</sup> ions is unaffected by the binding of bicarbonate (i.e., <i>K<sub>s</sub>S</i> ~ <i>K<sub>mS</sub></i>, and <i>K<sub>s</sub>A</i> ~ <i>K<sub>mA</sub></i>).

Similar analyses with respect to ATP, illustrate a different activation behavior by K<sup>+</sup> (Fig. 3). Lineweaver-Burk analysis of the effect of increasing ATP on MCCase activity, in the presence of different concentrations of K<sup>+</sup> ions, indicate that the enzyme is activated by a mixed mechanism relative to ATP (Fig. 3B). That is, K<sup>+</sup> ions increase <i>V<sub>max</sub></i>, and decrease
Table I. Kinetic constants for the activation of MCCase by K⁺.

<table>
<thead>
<tr>
<th>Kinetic Constant</th>
<th>Substrate</th>
<th>ATP</th>
<th>HCO₃⁻</th>
<th>Mc-CoA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_S^S$</td>
<td>65.9 µM</td>
<td>1.02 mM</td>
<td>19.4 µM</td>
<td></td>
</tr>
<tr>
<td>$K_m^S$</td>
<td>31.0 µM</td>
<td>0.96 mM</td>
<td>37.6 µM</td>
<td></td>
</tr>
<tr>
<td>$K_S^A$</td>
<td>1.15 mM</td>
<td>6.10 mM</td>
<td>3.3 mM</td>
<td></td>
</tr>
<tr>
<td>$K_m^A$</td>
<td>0.54 mM</td>
<td>5.74 mM</td>
<td>6.4 mM</td>
<td></td>
</tr>
<tr>
<td>k'/k</td>
<td>4.61</td>
<td>1.89</td>
<td>1.94</td>
<td></td>
</tr>
</tbody>
</table>

*Mc-CoA = methylcrotonyl-CoA
Figure 2. Activation of maize MCCase by K⁺, relative to ATP. The purified MCCase preparation was assayed by the procedure described under Materials and Methods. (A) Effect of increasing concentration of K⁺ on MCCase activity. MCCase was assayed at the indicated concentrations of ATP in the presence of 0 mM (●), 2 mM (□), 5 mM (○), 10 mM (×), 20 mM (▲), and 30 mM (○) K⁺. (C) Secondary plot of the reciprocals of Δ y-intercept and Δ slope of the Lineweaver-Burk lines versus the reciprocal of K⁺ concentration.
Figure 3. Activation of maize MCCase by K+, relative to ATP. The purified MCCase preparation was assayed by the procedure described under Materials and Methods and as modified below. (A) Effect of increasing concentration of KCl on MCCase activity. MCCase was assayed at the indicated concentrations of ATP in the presence of 0 mM (○), 2 mM (□), 5 mM (▲), 10 mM (●), 20 mM (■), and 30 mM (▲) K+. (B) Lineweaver-Burk analysis of the data in panel A. (C) Secondary plot of the reciprocals of Δ y-intercept and Δ slope of the Lineweaver-Burk lines versus the reciprocal of K+ concentration.
Figure 4. Activation of maize MCCase by K⁺, relative to Mc-CoA. The purified MCCase preparation was assayed by the procedure described under Materials and Methods and as modified below. (A) Effect of increasing concentration of K⁺ on MCCase activity. MCCase was assayed at the indicated concentrations of ATP in the presence of 0 mM (○), 2 mM (□), 5 mM (●), 10 mM (▲), and 30 mM (△) K⁺. (C) Secondary plot of the reciprocals of y-intercept and slope of the Lineweaver-Burk lines versus the reciprocal of K⁺ concentration.
the value of $K_m$ for ATP. By analysing the ATP data as described above for Scheme 1, the values of the dissociation constants for the various enzyme, substrate and activator complexes were calculated from Figure 3C and presented in Table I. The finding that $K_{s,S} > K_{m,S}$, indicates that the substrate, ATP, prefers to bind to the enzyme-activator complex. Analogously, $K_{s,A} > K_{m,A}$, therefore, $K^+$ ions prefer to bind to the enzyme-ATP complex. These data indicate that $K^+$ ions and ATP facilitate each others binding to MCCase.

Finally, the activating effect of $K^+$ ions was investigated relative to the substrate methylcrotonyl-CoA (Fig. 4). Lineweaver-Burk analysis of these data presented in Figure 4A suggest that $K^+$ ions activate MCCase by an uncompetitive kinetic mechanism relative to methylcrotonyl-CoA. That is, $K^+$ increases the $V_{\text{max}}$ of the enzyme, and increases the $K_m$ value for methylcrotonyl-CoA. The values of the dissociation constants for Scheme I, $K_{s,S}$, $K_{m,S}$, $K_{s,A}$ and $K_{m,A}$, were obtained with the use of secondary plots, as described above, with one exception. Since, the Lineweaver-Burk plots generate a family of parallel lines, the value of $\Delta_{\text{slope}}$ at each $K^+$ concentration is zero, therefore, $1/\Delta_{\text{slope}} = \infty$. Therefore, the $y$-intercept for the $\Delta_{\text{slope}}$ secondary plot, $k_{\text{el}}(K_{s,S} - K_{m,S}.k/k') = \infty$, which implies that $K_{s,S} = K_{m,S}.k/k'$, and thus, $K_{s,A}/K_{m,A} = k/k'$. As pointed out by Frieden (1964), a mixed type of mechanism in which, $K_{s,A}/K_{m,A} = k/k'$, will give rise to uncompetitive kinetics. Thus, the activation of MCCase by $K^+$, relative to methylcrotonyl-CoA, is mixed, and the calculated value of $K_{s,A}/K_{m,A} = k/k' = 0.52$ (Table I). The calculated values of the dissociation constants for the enzyme, methylcrotonyl-CoA and $K^+$ complexes are given in Table I. These values indicate that binding of $K^+$ and methylcrotonyl-CoA is antagonistic, that is, $K^+$ prefers to bind to the enzyme, prior to the binding of methylcrotonyl-CoA, and conversely methylcrotonyl-CoA prefers to bind to the enzyme prior to the binding of $K^+$.

In all these analyses, the ratio of $k'/k$ was determined with respect to the three substrates, bicarbonate, ATP and methylcrotonyl-CoA. As expected, the value of these ratios
were >1 and ranged from 1.89 to 4.61, indicating that the rate of product appearance is higher when K⁺ is bound to the enzyme-substrate complexes.

In summary, the kinetic analyses of the activation of MCCase by K⁺ indicate that this activator does not affect the binding of the substrate bicarbonate. However, K⁺ affects the binding of the other two substrates, ATP and methylcrotonyl-CoA. K⁺ is a positive effector of the binding of ATP, lowering the dissociation constant for the enzyme-ATP complex. In contrast, K⁺ is a negative effector of the binding methylcrotonyl-CoA, increasing the dissociation constant for the enzyme-methylcrotonyl-CoA complex. Since ATP and methylcrotonyl-CoA are thought to bind to physically separate sites on the enzyme, the biotin carboxylase and carboxyltransferase active subsites, the data presented in this publication could be interpreted to indicate that K⁺ can bind to both subsites. Alternatively, K⁺ binds to one subsite, and this causes a conformational change in the enzyme that affects the binding of the other substrate. We suggest that this later hypothesis is more likely to be correct, and that the K⁺ binding site is in the ATP-binding, biotin carboxylase subsite. The rationale for supporting this hypothesis is based upon a survey of enzymes that are activated by monovalent cations (Suelter, 1970). One of the common features between the mechanisms of these enzymes and the mechanism of MCCase, is the formation of the intermediate with the following general structure:

\[
\begin{array}{c}
\text{X} \\
\text{II} \\
\text{R - C - Y - R}
\end{array}
\]

where X is O, N, or C and where Y is O or N. Such an intermediate occurs during the carboxylation of biotin, that is the formation of carboxyphosphate, the activated form of bicarbonate, which reacts with biotin to form N-1 carboxybiotin (Knowles, 1988). The function of K⁺ in this reaction would be to act as a bridge between the enzyme and the carboxyphosphate intermediate (Suelter, 1970).
References

PURIFICATION AND KINETIC STUDIES OF 3-METHYLCROTONYL-CoA CARBOXYLASE FROM Glycine max

A paper to be submitted to the Biochimica et Biophysica Acta

Jianping Song, Tomás A. Diez, Tuan-Nam Wen, Yang Chen, Eve S. Wurtele and Basil J. Nikolau

Abstract

3-Methylcrotonyl-CoA carboxylase was purified from soybean seedlings to near homogeneity. The enzyme is composed of an 85 kDa biotin-containing subunit and a 59 kDa biotin-free subunit. These two subunits appear to be present in an equal molar ratio. Like the animal MCCase, the soybean MCCase probably has an α6β6 quaternary structure. The kinetics of the purified enzyme were studied. Based on the results of initial velocities and inhibition patterns of the competitive inhibitors of 3-methylcrotonyl-CoA carboxylase, a random Bi Bi Uni Uni Ping Pong mechanism is proposed. Such a mechanism has also been observed for acetyl-CoA carboxylase from castor oil seeds (Finlayson et al., 1983) and pyruvate carboxylase from chicken liver (Barden et al., 1972). An initial rate equation for the proposed mechanism was developed by utilizing a computer-assisted derivation of the steady-state equation method. The kinetic constants have been calculated from secondary plots.
Introduction

3-Methylcrotonyl-CoA carboxylase (MCCase, EC 6.4.1.4) is a member of a group of enzymes that utilize biotin in their catalytic mechanism. MCCase catalyzes the ATP-dependent carboxylation of 3-methylcrotonyl-CoA (Mc-CoA) to form 3-methylglutaconyl-CoA (Moss and Lane, 1971; Wood and Harden, 1977). The reaction catalyzed by MCCase is required in the catabolism of leucine to acetoacetyl-CoA and acetyl-CoA (Coon, et al., 1959; Rodwell, 1969).

A common feature in the reactions catalyzed by biotin enzymes is the transfer of a carboxyl group from one substrate to another. Various kinetic studies (reviewed by Moss and Lane, 1971; Knowles, 1989) suggest that biotin enzymes catalyze two-step reactions: the first being the carboxylation of the biotin prosthetic group (Reaction [1]), and the second being the transfer of the carboxyl group from the carboxy-biotin intermediate to an acceptor molecule (Reaction [2]). Overall these two half-reactions are sum to Reaction [3]. These studies have indicated that the kinetic mechanism of biotin enzyme is a Bi Bi Uni Uni Ping Pong according to the terminology of Cleland (1963).

\[
\begin{align*}
\text{Mg}^{2+} & \quad \text{ATP} + \text{HCO}_3^- + \text{E-biotin} \rightarrow \text{ADP} + \text{Pi} + \text{E-biotin-CO}_2^- \quad [1] \\
\text{E-biotin-CO}_2^- + \text{Acceptor} \rightarrow \text{E-biotin} + \text{Acceptor-CO}_2^- \quad [2] \\
\text{Mg}^{2+} & \quad \text{sum:} \quad \text{ATP} + \text{HCO}_3^- + \text{Acceptor} \rightarrow \text{ADP} + \text{Pi} + \text{Donor} + \text{Acceptor-CO}_2^- \quad [3]
\end{align*}
\]

In addition, the structural organizations of \textit{E. coli} acetyl-CoA carboxylase (Alberts et al., 1969; Guchhait et al., 1974a; 1974b; Polakis et al., 1974) and transcarboxylase from \textit{Propionibacteria} (Chung et al., 1975; Wood and Zwolinski, 1976; Wood and Kumar, 1985) have provided evidence that each half reaction occurs at physically separate active sites. In
In this paper, we report kinetic studies on the substrate interactions and patterns of inhibition by competitive inhibitors of MCCase. The results are consistent with soybean MCCase following a random Bi Bi Uni Uni Ping Pong mechanism.

Materials and Methods

Reagents - Q-Sepharose Fast Flow was obtained from Pharmacia LKB Biotechnology Company. Avidin was purchased from Pierce Chemical Co. Agarose Cibacron Blue 3GA, cyanogen bromide-activated Sepharose 4B and 3-methylcrotonyl-CoA were purchased from Sigma Chemical Company. [14C]-NaHCO₃ (58 μCi/μmol) was from Amersham Life Science. The monomeric avidin affinity column was prepared as described by Henrikson et al. (1979). All other reagents were from United States Biochemicals.

Plant Material - Soybean (Glycine max cv Corsoy 79) seeds were germinated in a greenhouse at 25°C. Plants were watered daily.

Purification of MCCase - All procedures were performed at 4°C. Five-day-old soybean seedlings were pulverized in a mortar with a pestle in the presence of liquid N₂. The frozen, ground tissue was homogenized with 3-4 volumes of buffer containing 0.1 M Hepes-KOH, pH 7.0, 1 mM EDTA, 0.1% (v/v) Triton X-100, 20% (v/v) glycerol, 20 mM β-mercaptoethanol, 100 μg/ml PMSF, 10 μM trans-epoxysuccinyl-L-leucylamido(4-guanidone)-butane. The extract was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 22,100g for 15 min. To the resulting supernatant, PEG-8000 was added to a final concentration of 18%. Precipitated proteins were collected by centrifugation at 22,100g for 15 min, and the pellet was dissolved in the minimal volume of Buffer A. Buffer A was composed of 10 mM Hepes-KOH, pH 7.0, 20 mM β-mecaptoethanol, 20% (v/v) glycerol. The enzyme preparation obtained from the PEG precipitation was passed through an Agarose Cibacron Blue 3GA column (2 cm X 10 cm). The column was washed...
with 500-1000 ml buffer A to remove nonspecifically binding proteins. MCCase was eluted with a linear gradient of 0-1 M KCl in buffer A. All the fractions containing MCCase activity were pooled, dialyzed against buffer A, and passed through a Q-Sepharose column (3 cm x 20 cm), pre-equilibrated with buffer A. The column was then washed with 500 ml of buffer A to remove nonspecifically binding proteins. MCCase was then eluted with a linear gradient of 0-0.8 M KCl in buffer A. All the fractions containing MCCase activity were pooled, dialyzed against buffer A, and passed through an Agarose-monomeric avidin affinity column (3 cm x 20 cm), pre-equilibrated with buffer A. The column was washed with buffer A containing 0.25 M KCl and MCCase was eluted with buffer A containing 0.25 M KCl and 0.4 mM biotin.

**Enzyme Assays** - MCCase activity was assayed by the incorporation of radioactivity from $\text{H}^{14}\text{CO}_3^-$ into the acid-stable product. The reaction mixture containing 0.1 M Tricine-KOH, pH 8.0, 5 mM MgCl$_2$, 2.5 mM DTT, various amounts of bicarbonate ($[\text{KHCO}_3]/[\text{NaH}^{14}\text{CO}_3] = 10$), ATP, and 3-methylcrotonyl-CoA in a final volume of 200 μl. The assay was initiated by the addition of the enzyme, and incubated at 37°C for 10 min. The reaction was then terminated by the addition of 50 μl 6 N HCl. An aliquot (100 μl) was dried on a strip of Whatman 3MM paper, and the acid-stable radioactivity was determined by liquid scintillation counting.

**Protein Determination** - Protein concentration was determined by the method of Bradford (1972).

**Electrophoresis and Western Blot Analysis** - SDS-PAGE was performed in 7.5% acrylamide gels as described previously (Laemmli, 1970). Following electrophoresis, proteins were electrophoretically transferred to a nitrocellulose membrane (Kyhse-Andersen, 1984), and the biotin-containing proteins were specifically detected using $^{125}\text{I}$-streptavidin (Nikolau et al., 1985). Immunological detection was performed by sequential incubation of
the filter with polyclonal antibodies, diluted between 1:500 and 1:2000, and with 125I-Protein A.

Results

Purification of MCCase - In previous studies on the distribution of MCCase activity among various organs of soybean, we had determined that germinating soybean seedlings, at an early stage of seedling development, have high MCCase activity (Song et al., submitted for publication). Therefore, 5-day-old soybean seedlings were used as the tissue source for the purification of MCCase. MCCase was isolated and purified by a five-step purification procedure outlined in Table I.

Proteins, extracted from soybean seedlings, were collected and concentrated by precipitation at 18% PEG-8000. Nearly 90% of the MCCase activity was recovered in the 18% PEG precipitate. This fraction was then subjected to affinity chromatography on an Agarose Cibacron Blue 3GA column, which resulted in a high recovery of MCCase activity, with about a 7-fold purification. The MCCase recovered from the Cibacron Blue column was subsequently purified on a Q-Sepharose ion exchange column, followed by affinity chromatography on a monomeric avidin column. Ion exchange chromatography did not give a significant increase in the specific activity of the preparation, however, it was retained in the procedure since it concentrated the enzyme sample, which helped in the subsequent purification step. The last affinity chromatographic step yielded a MCCase preparation with a specific activity of nearly 340 nmol/min-mg protein, with about 46% recovery of the activity as compared with the initial crude extract.

The purified MCCase preparation was analyzed by SDS-PAGE and the resulting gel was either stained with Coomassie Brilliant Blue or subjected to western blot analyses. Two
<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units/mg)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>714.3</td>
<td>662.9</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>0-18% PEG</td>
<td>594.5</td>
<td>594.5</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Cibacron Blue</td>
<td>105.7</td>
<td>724.3</td>
<td>6.8</td>
<td>7.6</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>80.1</td>
<td>548.7</td>
<td>6.9</td>
<td>7.7</td>
</tr>
<tr>
<td>Monomeric Avidin</td>
<td>0.9</td>
<td>302.9</td>
<td>336.6</td>
<td>374.0</td>
</tr>
</tbody>
</table>

*a350 g of soybean seedlings were used.  
1 unit = 1 nmol of HCO₃⁻ incorporated into 3-methylglutaconyl-CoA per minute.*
protein bands were present in the enzyme preparation with molecular weights of 85,000 and 59,000 (Fig. 1A). Densitometric scans of these gels indicate that these two polypeptides are present in equimolar ratio. Western blot analyses with 125I-streptavidin (Fig. 1B) or antiserum (Fig. 1C) against the biotin-containing subunit of MCCase (Song et al., submitted for publication) revealed that 85 kDa polypeptide contains biotin and is the biotin-containing subunit of MCCase of soybean. The 59 kDa polypeptide is the biotin-free subunit of MCCase. We have previously reported that the molecular weight of the native soybean MCCase is 970,000 ± 83,000 (Song et al., submitted for publication). Therefore, these results suggest that the soybean MCCase has probably an α6β6 quaternary structure.

**Initial Velocity Studies** - Cleland (1963) and Fromm (1967) have described the potential kinetic mechanisms for enzymes that utilize three substrates. These mechanisms fall into two classes, Sequential and Ping Pong. Two kinetic approaches have been used to identify the specific kinetic mechanism a reaction is obeying. One approach is to hold one substrate at a constant saturating concentration, measuring the initial velocity upon varying the concentrations of the other two substrates (Frieden, 1959). In essence the enzyme is treated as a two-substrate system. Another approach is to vary the concentration of one substrate at different concentrations of the other two substrates, while retaining a constant ratio between these later two substrates (Fromm, 1967). This analysis is then repeated for all three substrates. This later approach was adopted in the studies of the kinetic mechanism of soybean MCCase.

In a Sequential mechanism, the double reciprocal plots of velocity against each substrate concentration will yield a series of converging lines. However, in a Ping Pong mechanism, one or more of the double reciprocal plots of the velocity against each of the substrate concentrations will give rise to a series of parallel lines. Double reciprocal plots of initial-rate data for MCCase for each substrate are illustrated in Fig. 2. Two of the plots obtained from these analyses yield a series of intersecting lines; the plots of the reciprocal of
Figure 1. Analysis of the purified soybean MCCase. MCCase, purified as described in the Materials and Methods, was subjected to SDS-PAGE and the resulting gel was stained with Coomassie Brilliant Blue (A). An identical gel was subjected to Western analysis with $^{125}$I-streptavidin (B), or immunological detection with antibodies to the soybean MCCase and $^{125}$I-Protein A (C).
Figure 2. Initial-rate studies of MCCase. A. Plot of reciprocal of initial velocity with respect to the reciprocal of the molar concentration of ATP, at the indicated concentration of Mc-CoA and a ratio of [HCO₃⁻]/[Mc-CoA] = 100. The inset is secondary plots of the intercept and/or slope derived from the primary plots with respect to the concentration of Mc-CoA. B. Plot of reciprocal of initial velocity with respect to the reciprocal of the molar concentration of bicarbonate, at the indicated concentration of ATP and a ratio of [ATP]/[Mc-CoA] = 1. The inset is secondary plots of the intercept and/or slope derived from the primary plots with respect to the concentration of ATP. C. Plot of reciprocal of initial velocity with respect to the reciprocal of the molar concentration of Mc-CoA at the indicated concentration of bicarbonate and a ratio of [HCO₃⁻]/[ATP] = 50. The inset is secondary plots of the intercept and/or slope derived from the primary plots with respect to the concentration of bicarbonate.
initial velocity with respect to the reciprocal of the concentration of ATP (Fig. 2A) or bicarbonate (Fig. 2B) generate a series of converging lines that intercept to the left of the y-axis. However, the plot of the reciprocal of initial velocity with respect to the reciprocal of the Mc-CoA concentration generates a series of parallel lines (Fig. 2C). Therefore, these data eliminated all Sequential mechanisms for the reaction catalyzed by MCCase. In addition, the Hexa Uni Uni Ping Pong mechanism which uniquely generates sets of parallel lines with each of the three substrates is eliminated in the case of MCCase.

There are potentially eight Ping Pong mechanisms that can describe the MCCase reaction; these are the Random or Ordered Bi Bi Uni Uni, Uni Uni Bi Bi, Bi Uni Uni Bi, and Uni Bi Bi Uni. From many previous studies of biotin enzymes (Moss and Lane, 1971; Knowles, 1989), including MCCase from *Achromobacter* (Schiele et al., 1975), that it is obvious the first half reaction catalyzed by biotin enzymes is a Bi Bi Uni Uni reaction in which ATP and bicarbonate react as the first two substrates to generate the first two products, ADP and phosphate. Indeed, secondary plots constructed from the primary double-reciprocal data are consistent with a Bi Bi Uni Uni mechanism for MCCase. For each set of data, the slope or y-intercept of each line from the primary data was plotted against the concentration of the non-varying substrate (see insets of Fig. 2). When ATP or bicarbonate were the variable substrates, secondary plots generated linear lines (see insets Fig. 2A and Fig. 2B, respectively). However, secondary replots of the primary reciprocal data generated with variable concentrations of Mc-CoA gives rise to a non-linear line.

Both Ordered and Random Bi Bi Uni Uni Ping Pong mechanisms can be mathematically described by the same (Equation [1]) (Fig. 3) and therefore can not be distinguished purely from initial velocity studies. However, these two mechanisms can be distinguished from each other by observing either the patterns of inhibitions of competitive inhibitors of each substrate or the inhibition patterns by products of the reaction (Fromm,
\[
\frac{1}{v} = \frac{1}{V_m} \cdot \left( \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_c}{C} + \frac{K_{ab}}{AB} \right)
\]  

Scheme I. Ordered Bi Bi Uni Uni Ping Pong mechanisms.

\[
\frac{V_m}{v} = 1 + \frac{k_{11}}{k_{3}} + \frac{k_{11}}{k_{1}} + \frac{k_{11}(k_{4} + k_{5})}{k_{k_{15}}} + \frac{1}{A} + \frac{k_{10} + k_{11}}{k_{9}} + \frac{k_{k_{15}}(k_{4} + k_{11})}{k_{k_{15}} \times AB}
\]

Scheme II. Random Bi Bi Uni Uni Ping Pong mechanism.

\[
\frac{V_m}{v} = 1 + \frac{k_{19}}{k_{9} + k_{13}} + \frac{k_{11}k_{19}}{k_{11}(k_{9} + k_{13})} + \frac{k_{k_{15}}}{k_{11}(k_{9} + k_{13})} + \frac{k_{k_{15}}(k_{9} + k_{3} + k_{9} + k_{13})}{(k_{9} + k_{13})(k_{10}k_{12} + k_{2}k_{3})} \times \frac{1}{A} + \frac{1}{B} + \frac{k_{18} + k_{19}}{k_{17}} + \frac{k_{k_{15}}(k_{9} + k_{3} + k_{9} + k_{13})}{k_{k_{15}} \times AB}
\]

<table>
<thead>
<tr>
<th>Vm</th>
<th>Ka</th>
<th>Kb</th>
<th>Kc</th>
<th>Kab</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 umol/min/mg</td>
<td>8.7 uM</td>
<td>618.5 uM</td>
<td>68.9 uM</td>
<td>37.4 mM</td>
</tr>
</tbody>
</table>

Kinetic constants for equation (3).

Figure 3. Schemes and initial velocity rate equations of Ordered or Random Bi Bi Uni Uni Ping Pong mechanisms.
We utilized competitive inhibitors of the substrates to distinguish these two mechanisms for MCCase.

To predict the patterns of inhibition of the competitive inhibitors of MCCase (Table II), the initial velocity rate equations for the two potential Ping Pong mechanisms were derived with steady state ENZ-EQ computer program described by Fromm (1975). In this derivation, A, B, C are the substrates, and P, Q, R are the products. Terms containing the concentration of P, Q, and R as products were eliminated, since only the initial velocity is considered. In addition, in the Random Bi Bi Uni Uni mechanism, it was assumed that $k_2 \gg k_3[A]$, and $k_6 \gg k_7[B]$. The initial velocity for the Ordered Bi Bi Uni Uni Ping Pong mechanism and the Random Bi Bi Uni Uni Ping Pong mechanism are expressed in equation [2] and equation [3] (Fig. 3), respectively.

Utilizing these two rate equations the types of inhibitions that would be generated by the competitive inhibitors of each substrate were predicted for each mechanism. In both Ordered and Random Bi Bi Uni Uni Ping Pong mechanisms competitive inhibitors of substrate A and C give identical patterns of inhibition with respect to the other two substrates and therefore are useless in distinguishing between these two mechanisms. However, a competitive inhibitor of the second substrate can distinguish between these two mechanisms since it acts as an uncompetitive inhibitor towards the first substrate in the Ordered mechanism, whereas it acts as a noncompetitive inhibitor in the Random mechanism. ADP was found to be a competitive inhibitor of ATP as indicated by Figure 4A. The $K_i$ value for ADP is 127.8 $\mu$M (Table III). However, ADP is a noncompetitive inhibitor with respect to HCO$_3^-$ ($K_i = 19.7$ mM) (Fig. 4B), and a uncompetitive inhibitor with respect to Mc-CoA ($K_i = 2.9$ mM) (Fig. 4C).

NaHSO$_3$ was found to be a competitive inhibitor of MCCase with respect to HCO$_3^-$ ($K_i = 2.4$ mM) (Fig. 5A), and a noncompetitive inhibitor with respect to ATP ($K_i = 8.0$ mM) (Fig. 5B). The observed patterns of inhibition by these two competitive inhibitors of
<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Competitive inhibitor for substrate</th>
<th>1/A plot</th>
<th>1/B plot</th>
<th>1/C plot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ordered Bi Bi Uni Uni Ping Pong</td>
<td>A</td>
<td>C</td>
<td>N</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>U</td>
<td>C</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>U</td>
<td>U</td>
<td>C</td>
</tr>
<tr>
<td>Random Bi Bi Uni Uni Ping Pong</td>
<td>A</td>
<td>C</td>
<td>N</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>N</td>
<td>C</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>U</td>
<td>U</td>
<td>C</td>
</tr>
</tbody>
</table>
Figure 4. Inhibition of MCCase by ADP. A. Plot of reciprocal of initial velocity with respect to the reciprocal of the molar concentration of ATP in the presence of indicated concentration of ADP. The inset is the slope derived from the primary plot with respect to the molar concentration of ADP. B. Plot of reciprocal of initial velocity with respect to the reciprocal of the molar concentration of bicarbonate in the presence of indicated concentration of ADP. The inset is the intercept derived from the primary plot with respect to the molar concentration of ADP. C. Plot of reciprocal of initial velocity with respect to the reciprocal of the molar concentration of Mc-CoA in the presence of indicated concentration of ADP. The inset is the intercept derived from the primary plot with respect to the molar concentration of ADP.
Figure 5. Inhibition of MCCase by HSO$_3^-$. A. Plot of reciprocal of initial velocity with respect to the reciprocal of the molar concentration of ATP in the presence of indicated concentration of bisulfate. The insets are the intercept derived from the primary plot with respect to the molar concentration of ADP. B. Plot of reciprocal of initial velocity with respect to the reciprocal of the molar concentration of bicarbonate in the presence of indicated concentration of bisulfate. The insets are the slope derived from the primary plot with respect to the molar concentration of ADP.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>Inhibition pattern</th>
<th>Ki</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>ATP</td>
<td>Competitive</td>
<td>127.8 uM</td>
</tr>
<tr>
<td></td>
<td>HCO$_3^-$</td>
<td>Noncompetitive</td>
<td>19.7 mM</td>
</tr>
<tr>
<td></td>
<td>Mc-CoA</td>
<td>Uncompetitive</td>
<td>2.94 mM</td>
</tr>
<tr>
<td>HSO$_3^-$</td>
<td>HCO$_3^-$</td>
<td>Competitive</td>
<td>2.35 mM</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>Noncompetitive</td>
<td>14.76 mM</td>
</tr>
</tbody>
</table>
MCCase are consistent with the predicted patterns of inhibition for the Random Bi Bi Uni Uni Ping Pong mechanism. In this mechanism, there is no preference for the order in which ATP and bicarbonate bind to the enzyme.

Discussion

MCCase was purified to near homogeneity from 5-day-old soybean seedlings by a five-step purification procedure. Purified MCCase is composed of a 85 kDa biotin-containing subunit and a 59 kDa biotin-free subunit. These two polypeptides appear to be present in equal molar ratio in a holoenzyme complex of 890 kDa (Song et al., submitted for publication). Thus soybean MCCase probably has an \( \alpha_6\beta_6 \) quaternary structure. The soybean MCCase resembles the bovine kidney MCCase, which is composed of a 80 kDa biotin-containing subunit and a 62 kDa biotin-free subunit and has an \( \alpha_6\beta_6 \) configuration (Lau et al., 1979). The eukaryotic MCCases appear to differ from the bacterial MCCases which are also composed of a biotin-containing subunit and a biotin-free subunit, but they are in an \( \alpha_4\beta_4 \) configuration (Schiele et al., 1975; Fall and Hector, 1977).

Initial velocity studies of the purified soybean MCCase, by using the method of Fromm (1975) are consistent with a Bi Bi Uni Uni Ping Pong kinetic mechanism for the reaction catalyzed by this enzyme. Additional kinetic studies of the effect of competitive inhibitors of the initial two substrates of MCCase (ATP and bicarbonate) established that the binding of ATP and bicarbonate to the enzyme occurs in random order. Therefore, the reaction catalyzed by MCCase follows a Random Bi Bi Uni Uni mechanism.

The mechanism deduced from the kinetic studies of the soybean MCCase is consistent with and extends previous biochemical studies of this enzyme from *Achromobacter* (Schiele et al., 1975). As with all biotin-dependent carboxylases, MCCase catalyzes a two step reaction mechanism, the first being the ATP-dependent carboxylation of
the enzyme bound biotin prosthetic group. In the case of the *Achromobacter* MCCase, the first half-reaction can be catalyzed by the biotin-containing subunit, independent of the biotin-free subunit (Schiele et al., 1975). The kinetic studies described herein indicate that the substrates of this half-reaction bind to the enzyme in a random fashion. Evidence that the biotin-containing subunit of the soybean MCCase can catalyze this first half-reaction comes from examination of the amino acid sequence of this subunit deduced from the nucleotide sequence of the cDNA clone coding for this protein (Song et al., submitted for publication). This sequence shows similarities to other biotin-containing enzymes in two domains. One domain at the carboxyl end of the protein which has a high degree of homology to the biotin carboxy-carrier protein (BCCP) of *E. coli* acetyl-CoA carboxylase is the carboxy-carrier domain, and the second domain at the amino terminal end of the protein which has a high degree of homology to the biotin carboxylase of *E. coli* acetyl-CoA carboxylase is the biotin carboxylase domain.

In the first-half reaction, in which ATP is hydrolyzed and biotin is carboxylated, probably via a carboxy-phosphate intermediate (Kaziro et al., 1962; Climent and Rubio, 1986; Tipton and Cleland, 1988; Ogita and Knowles, 1988; Phillips et al., 1992), the first two products, ADP and Pi are released from the enzyme. The "charged", carboxylated enzyme (E' in Scheme II) then reacts with Mc-CoA to form the final product 3-methylglutaconyl-CoA. This second half-reaction appears to be catalyzed by the biotin-free subunit of MCCase (Schiele et al., 1975).

The Bi Bi Uni Uni Ping Pong mechanism is shared by all biotin enzymes so far examined. However, the order in which the first two substrates bind appears to depend on the enzyme. The soybean MCCase, like chicken liver pyruvate carboxylase (Barden et al., 1972) and castor bean acetyl-CoA carboxylase (Finlayson and Dennis, 1983), binds to ATP and bicarbonate in a random order. However, rat liver acetyl-CoA carboxylase (Hashimoto and
Numa, 1971) and pyruvate carboxylase (Wallace et al., 1975) appear to bind these substrates in an ordered fashion, with ATP binding prior to the binding of bicarbonate.

References


Chung, M., Ahmad, F., Jacobson, B., and Wood H. G. Biochem. 14, 1611-1619


Fall, R. R., Hector, M. L. (1977) Biochem. 16, 4000-4005


Purification and biochemical characterization of propionyl-CoA carboxylase activity from Zea mays: Propionyl-CoA carboxylase activity in extracts of plants is a side reaction of acetyl-CoA carboxylase

A paper to be submitted to Archives of Biochemistry and Biophysics
Tomás A. Diez, Eve S. Wurtele and Basil J. Nikolau

Abstract

Propionyl-CoA carboxylase activity has been purified from maize leaves by hydrophobic interaction chromatography on Propyl-Agarose, affinity chromatography on Cibacron Blue 3GA-Agarose, and ion exchange chromatography on Q-Sepharose. During these purification procedures, acetyl-CoA carboxylase activity copurified with propionyl-CoA carboxylase. In the purified preparation, the specific activity of propionyl-CoA carboxylase and acetyl-CoA carboxylase was 120 and 165 nmol/min/mg, respectively, representing a 206- and 240-fold purification of each activity. Western blot and SDS-PAGE analyses of the purified preparation revealed the presence of two biotinylated polypeptides of about 200 and 220 kDa. These two biotin-containing polypeptides have been previously identified as components of maize leaf acetyl-CoA carboxylase (Egli et al., 1993). Mixed substrate kinetic analyses, using the purified enzyme preparation, indicate that the carboxylation of propionyl-CoA and acetyl-CoA were carried out by one enzyme. Consistent with this hypothesis, the carboxylation of propionyl-CoA and acetyl-CoA were identically inhibited by methylmalonyl-CoA (competitive inhibitor) and malonyl-CoA (competitive inhibitor), the respective products of each reaction. Furthermore, haloxyfop, a monocot specific herbicide inhibitor of acetyl-CoA carboxylase, inhibits by a mixed mechanism both carboxylation reactions. Both carboxylation activities are similarly
inhibited by haloxyfop when the enzyme source is the purified preparation or a crude extract. These data indicate that propionyl-CoA carboxylase activity detectable in extracts from plants is a side reaction of the acetyl-CoA carboxylase enzyme.

**Introduction**

Propionyl-CoA carboxylase (PCCase) is a biotin-containing enzyme catalyzing the ATP-dependent carboxylation of propionyl-CoA to form D-methylmalonyl-CoA. This reaction serves an anabolic function, providing precursors for the biosynthesis of multimethyl branched-chain fatty acids, as occurs in the uropygial glands of birds (Buckner and Kolattkudy, 1976), and in *Mycobacterium tuberculosis* (Rainwater and Kolattukudy, 1985) and in the biosynthesis of antibiotics (O’Hagan, 1991). In addition, the carboxylation of propionyl-CoA serves in the catabolism of odd-numbered or branched chain fatty acids, and in the catabolism of the amino acids valine, isoleucine and methionine (Moss and Lane, 1971). PCCases have been characterized from animal and microbial organisms, and the structure and regulation of these enzymes are well described (Moss and Lane, 1971).

Protein extracts from plants have been shown to support the ATP-dependent, avidin-sensitive carboxylation of propionyl-CoA (Wurtele and Nikolau, 1990), consistent with the presence of PCCase in these organisms. In order to characterize the structure and properties of, and begin to elucidate the metabolic function of PCCase in plants we undertook the purification of this enzyme from leaves of maize. This manuscript describes these characterizations.
Materials and Methods

Plant material. Maize seeds were planted and grown in a greenhouse at 30°C with a day-length, supplemented by artificial lighting, of 14h/day. Leaves were harvested 2 weeks after planting and immediately frozen in liquid N2.

Radioisotopes and biochemicals. The radiochemicals Na\[^{125}\text{I}\] (14.7 mCi/\(\mu\)g of iodine) and NaH\[^{14}\text{CO}_3\] (52.4 mCi/mmol) were purchased from Amersham. Q-Sepharose ion exchange resin was purchased from Pharmacia. The protein determining dye reagent was purchased from Bio-Rad. Avidin and PEG were purchased from USB. Streptavidin was purchased from Promega. Streptavidin was labeled with carrier-free Na\[^{125}\text{I}\] as described previously (Nikolau et al, 1985) to a specific radioactivity of 1.55 x 10^7 cpm/\(\mu\)g. Molecular weight protein standards were purchased from Gibco-BRL. Biochemicals were purchased from Sigma.

Extraction of PCCase. Maize leaves (500-1000 g), frozen in liquid N2, were pulverized in a mortar with a pestle. The resulting powder was homogenized at 4°C in a Waring blender for 2-3 minutes with 3-5 volumes of 0.1 M Hepes-KOH (pH 7.0), 20 mM 2-mercaptoethanol, 100 \(\mu\)g/ml PMSF, 0.1% (v/v) Triton X-100, 1 mM EDTA, and 20% (v/v) glycerol. The mixture was filtered through several layers of cheesecloth, and the filtrate was immediately centrifuged at 12,200 g (Sorval GS-3 rotor) for 30 min. The supernatant (crude extract) was recovered, and the pellet discarded.

Purification of PCCase. All the chromatographic steps were carried out at 4°C.

Step 1. Polyethylene glycol precipitation. Finely ground PEG 8000 was slowly added to the crude extract to a final concentration of 4g PEG/100 ml. The mixture was gently stirred until the PEG was completely dissolved. The suspension was then centrifuged at 12,200 g for 30 min. The supernatant was recovered and more PEG was added and dissolved to a final concentration of 16g PEG/100 ml. After the PEG was completely
dissolved, precipitated proteins were recovered by centrifugation. The pellet (4-16% PEG fraction) was retained and resuspended in Buffer A, which is composed of 10 mM Hepes-KOH (pH 7.0), 10 mM 2-mercaptoethanol, 1 mM EDTA, and 20% (v/v) glycerol.

Step 2. Hydrophobic interaction chromatography. The 4-16% PEG fraction was loaded on a Propyl-N-Agarose column (2.5 cm x 45 cm), previously equilibrated with Buffer A. The column was washed with Buffer A until the $A_{280}$ of the eluate was less than 0.05. PCCase was eluted with a linear gradient of 0 M-0.5 M KCl in Buffer A, at a flow rate of 0.5-1 ml/min. Fractions containing PCCase activity were pooled and kept at −20°C for no more than 1 day, prior to further purification.

Step 3. Affinity chromatography on Cibacron-Blue 3GA Agarose. The pooled fractions from the Propyl-N-Agarose column containing PCCase activity were applied to a column of Cibacron-Blue 3GA Agarose (2.5 cm x 10 cm) previously equilibrated with Buffer A. The column was washed with 5 column volumes of Buffer A. Elution of PCCase was accomplished with a linear gradient of 0 M-0.75 M KCl in Buffer A at a flow rate of 1-1.5 ml/min. Fractions containing PCCase activity were pooled and kept at −20°C for no more than 1 day, prior to further purification.

Step 4. Ion exchange chromatography on Q-Sepharose. The pooled fractions from the Cibacron-Blue affinity column were dialyzed against 4L of Buffer A for 5 h. The dialyzed PCCase preparation was then loaded on a Q-Sepharose column (2.6 cm x 18 cm) previously equilibrated with Buffer A. The column was washed with 10 volumes of Buffer A. Elution of PCCase was achieved with a 800 ml linear gradient of 0 M - 0.75 M KCl in Buffer A. Fractions containing PCCase activity were pooled and stored at −20°C.

Enzyme assay. PCCase and ACCase activities were determined as the propionyl-CoA- and acetyl-CoA-dependent rates of incorporation of radioactivity from NaH$^{14}$CO$_3$ into the acid-stable products (Wurtele and Nikolau, 1990). Unless otherwise stated, the assays routinely contained 10-60 nmol/min of the enzyme preparation, in a total volume of 200 µl.
that contained 50-100 mM Tricine-KOH (pH 8.0), 1 mM ATP, 5 mM MgCl₂, 1 mM DTT, 5.4 mM NaH¹⁴CO₃ (1.08 mCi/mmol), and 0.3 mM substrate (acetyl-CoA or propionyl-CoA). The reaction was incubated at 37°C for 10 min and subsequently stopped by the addition of 50 µl of 6 N HCl. A 100 µl aliquot of the reaction mixture was spotted and dried on a piece of Whatmann 3MM paper, and the incorporated radioactivity was determined with a Packard Liquid Scintillation Analyzer. Control assays contained no acyl-CoA substrates.

**Analytical methods.** The method of Bradford (1976) was used to determine protein concentration. The concentrations of propionyl-CoA and acetyl-CoA were determined by the method of Lipmann and Tuttle (1945).

**Electrophoresis and western blotting analysis.** The enzyme preparations were analyzed by polyacrylamide gel electrophoresis in denaturing conditions. In doing so, protein samples were adjusted to 50 mM Tris pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM dithiothreitol, and placed in a boiling bath for 5 min. Proteins were fractionated by electrophoresis in polyacrylamide slabs (16 cm x 18 cm) composed of 5% (w/v) acrylamide/0.26% (w/v) bisacrylamide and containing 0.1% SDS (Laemmli, 1970). After electrophoresis, the gels were stained with Coomassie Brilliant Blue, or subjected to western blot analysis (Kyhse-Andersen, 1984). Biotin-containing polypeptides were detected by incubating the blots with ¹²⁵I-streptavidin (Nikolau et al., 1985).

**Results**

**Purification of PCCase.** This paper reports the isolation and purification of a PCCase activity from maize leaves. Before adopting the final purification procedure, studies on the extraction and stabilization of PCCase were conducted. These studies revealed that the extraction of PCCase activity is most efficient at pH 7.0, and that this activity is precipitated in the fraction between 4% and 16% PEG. The addition of 20% glycerol in the buffer
solution was strictly necessary to stabilize the enzyme (data not shown). In buffers containing glycerol and at pH 7.0 PCCase preparations could be stored for at least 2 months at -20°C without any appreciable loss of activity.

The results of a representative purification experiment are summarized in Table I. PCCase was purified over 200-fold, to a final specific activity of 120 nmol/min.mg protein. During the purification, partial separation of PCCase from MCCase was obtained by hydrophobic interaction chromatography on Propyl-Agarose (data not shown). However, the elution of PCCase activity from this column coincided with ACCase (Figure 1A). A complete removal of MCCase from the PCCase preparation was obtained when the Propyl-Agarose pooled fraction was further purified through affinity chromatography on Cibacron-Blue 3GA (data not shown). However, once again, both PCCase and ACCase coeluted from the Cibacron-Blue 3GA column (Figure 1B). Further purification of PCCase was achieved by ion exchange chromatography on Q-Sepharose, although recovery from this step was low. Once again, elution of PCCase activity coincided with the elution of ACCase (Figure 1C). Table I illustrates the copurification of ACCase that was achieved when PCCase activity was being purified. Through the four purification steps used in this procedure PCCase and ACCase purification was coincidental. This is best illustrated by the PCCase/ACCase ratio in the five purification fractions, which ranged between 0.5 to 0.71 (Table I).

SDS-PAGE analysis of the purified PCCase fraction revealed the presence of only 2 polypeptides of approximately 200 kDa and 220 kDa (Figure 2A). Western blot analysis with 125I-streptavidin of a replica of the same gel showed that both these polypeptides were biotinylated (Figure 2B). The same sized biotinylated polypeptides have been purified and identified as subunits of ACCase (Egli, et al., 1993). Whereas, the biotin-containing subunit of PCCases purified previously from animal and microbial sources have molecular weights of between 64 kDa and 72 kDa (Henrikson and George Allen, 1979; Kalousek, et al., 1980).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Volume (ml)</th>
<th>Total Protein [mg]</th>
<th>Total Activity (nmol/min)</th>
<th>Recovery %</th>
<th>Specific Activity (nmol/min.mg)</th>
<th>Purification-fold</th>
<th>PCCase: ACCase ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>2400</td>
<td>5040</td>
<td>4056 2496</td>
<td>100.0 100.0</td>
<td>0.50 0.80</td>
<td>1.0 1.0</td>
<td>0.63</td>
</tr>
<tr>
<td>4-16% PEG</td>
<td>198</td>
<td>1295</td>
<td>3099 1653</td>
<td>66.2 76.4</td>
<td>1.28 2.39</td>
<td>2.6 3.0</td>
<td>0.53</td>
</tr>
<tr>
<td>Propyl-Agarose</td>
<td>272</td>
<td>438</td>
<td>3633 1784</td>
<td>71.5 89.6</td>
<td>4.07 8.30</td>
<td>8.1 10.4</td>
<td>0.49</td>
</tr>
<tr>
<td>Cibacron-Blue</td>
<td>170</td>
<td>106</td>
<td>4306 2556</td>
<td>102.4 106.2</td>
<td>24.1 40.5</td>
<td>48.1 50.6</td>
<td>0.60</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>19</td>
<td>3</td>
<td>502 365</td>
<td>14.6 12.4</td>
<td>120.0 165.0</td>
<td>240.0 206.3</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*1 kg of plant tissue*
Figure 1. Elution profile of PCCase/ACCase on Propyl-Agarose (A), Cibacron-Blue 3GA (B), and Q-Sepharose (C).
Figure 2. SDS-PAGE and western analysis of the purified PCCase/ACCase preparation. PCCase activity was purified as described in the Materials and Methods. An aliquot of the purified preparation was subjected to SDS-PAGE, and the resulting gel stained with Coomassie Brilliant Blue (A). An identical gel was subjected to western analysis, and biotin-containing polypeptides were detected with ^125I-streptavidin.
No biotin-containing polypeptide in this size range was found in our preparation. These results can be explained by two hypotheses, either the PCCase activity found in plant extracts is a side reaction of the ACCase enzyme, or PCCase is a distinct enzyme, which could not be detected by the western blot analyses, and it copurifies with ACCase through the purification scheme described. To distinguish between these two hypotheses, the following comparative kinetic characterizations of the purified PCCase/ACCase preparation were conducted.

**Mixed substrate studies.** Based on the kinetic studies of Thorn (1949) and Whittaker and Adams (1949), Webb and Morrow (1959) developed a kinetic method to determine whether a single enzyme catalyzes the conversion of two different substrates. This approach, which is called the "mixed substrate method", compares the rate of reaction on an equimolar mixture of the two substrates relative to that of the reactions with each of the individual substrates. If different enzymes are attacking the two substrates, then the maximum velocity with a mixture of substrates will be the sum of the maximum velocities obtained with each individual substrate. On the other hand, if the same enzyme attacks both substrates, the maximum obtainable velocity with a mixture of substrates will fall in between the values of the maximum velocities for each single substrate, since both substrates compete for the same active site. This kinetic behavior is described by the following equation (Webb and Morrow, 1959):

\[
V_t = \frac{V_a \alpha + V_b \beta}{1 + \alpha + \beta}
\]

where \(\alpha\) is the concentration of one substrate (a) and \(\beta\) is the concentration of the second substrate (b), and \(V_t\), \(V_a\), and \(V_b\) are the maximum velocities in the presence of both substrates, substrate a, and substrate b, respectively.

If the analyses are carried out at relatively equal concentrations of a and b, so that \(\alpha = \beta = \sigma\), then
Thus, at low concentrations of the substrates,

\[ V_t = V_a + V_b \]

However, \( V_t \) will asymptotically approach \( (V_a + V_b)/2 \) as the concentration of the substrates is increased and approach infinity (Webb and Morrow, 1959).

Using the purified PCCase/ACCase preparation we determined the rates of carboxylation with increasing concentrations of propionyl-CoA (\( V_p \)) or acetyl-CoA (\( V_a \)) or equimolar mixture of propionyl-CoA and acetyl-CoA (\( V_t \)). As shown in Figures 3A, the rate of carboxylation with the equimolar mixture was lower than the rate of carboxylation with acetyl-CoA, but higher than that with propionyl-CoA. As predicted for a single enzyme catalyzing these carboxylation reactions, the value of \( V_t/V_a + V_b \) fell from 0.94 to 0.59 as \( \sigma \) was increased from 10 \( \mu \)M to 750 \( \mu \)M (Figure 3B). These data indicate that in the purified PCCase/ACCase preparation a single enzyme is catalyzing the carboxylation of propionyl-CoA and acetyl-CoA.

The same analyses were conducted using a crude extract as the source of the enzyme. Figures 4A and 4B show the results that were obtained. As with the purified enzyme preparation, the carboxylation rate with the propionyl-CoA acetyl-CoA mixture was less than that obtained with acetyl-CoA alone, but greater than that obtained with propionyl-CoA alone. Most significantly, the data points of the plot of \( V_t/(V_a + V_b) \) versus substrate concentration closely matched that predicted for the carboxylation of propionyl-CoA and acetyl-CoA by a single enzyme. Thus, in both a crude extract and purified enzyme preparation, the carboxylation of propionyl-CoA and acetyl-CoA appear to be catalyzed by a single enzyme.
Figure 3. Mixed substrate analysis of PCCase and ACCase activities. A) The rate of carboxylation of propionyl-CoA (V_P), acetyl-CoA (V_A) and an equimolar mixture of propionyl-CoA and acetyl-CoA was determined as described in Materials and Methods. B) From the data presented in panel A, V_t/V_A + V_P is plotted against the substrate concentrations. Source of the enzyme was the purified PCCase/ACCase preparation.
Figure 4. Mixed substrate analysis of PCCase and ACCase activities. A) The rate of carboxylation of propionyl-CoA (VP), acetyl-CoA (VA) and an equimolar mixture of propionyl-CoA and acetyl-CoA was determined as described in Materials and Methods. B) From the data presented in panel A, $V_t/VA + VP$ is plotted against the substrate concentrations. Source of the enzyme was the 4-16% PEG fraction.
Inhibition studies. If propionyl-CoA and acetyl-CoA are carboxylated by the same enzyme, we would expect that each reaction will be affected by the same inhibitors and each inhibitor would show the same inhibition pattern. In addition, if all the PCCase activity in the plant is due to a side reaction of the ACCase enzyme, the extent of inhibition of each reaction will be the same when assayed in a crude extract and in the purified enzyme preparation. We chose three inhibitors to test with the PCCase/ACCase reactions. These being the herbicide haloxyfop, which is a potent inhibitor of ACCase from monocot plants (Burton et al., 1989), and the carboxylated products of ACCase and PCCase, malonyl-CoA and methylmalonyl-CoA, respectively.

In crude extracts, the carboxylation of propionyl-CoA was inhibited by haloxyfop, with 50% inhibition occurring at 0.45 μM (Figure 5A). Similarly, the carboxylation of acetyl-CoA was inhibited by haloxyfop with 50% inhibition occurring at 1.78 μM (Figure 5A). These two reactions were similarly inhibited by haloxyfop when assayed with the purified PCCase/ACCase preparation (Fig. 6). In all these inhibition studies, the effect of increasing haloxyfop concentration inhibited almost identically the carboxylation of propionyl-CoA and acetyl-CoA (cf., Figs. 5A to 5B and 6A to 6B).

Further kinetic studies revealed that haloxyfop inhibits the carboxylation of both propionyl-CoA and acetyl-CoA through a mixed mechanism (Figures 6A and 6B). The values of the inhibition constants were determined by replotting the slopes and intercepts from the Lineweaver-Burk plots versus the concentration of haloxyfop (Insets of Figures 6A and 6B, and Table II). These experiments clearly demonstrate that haloxyfop is a more potent inhibitor of the carboxylation of propionyl-CoA than acetyl-CoA (cf., $K_i$ and $K'_i$ values for each reaction, Table II). This can be rationalized in terms of the structural similarities between haloxyfop and propionyl-CoA around the reacting carbon atom (Figure 7). In addition, $K_i$s are lower than the respective $K'_i$s indicating that haloxyfop is a more potent inhibitor with the free enzyme than when the enzyme has bound the substrate.
Figure 5. Effect of haloxyfop on maize PCCase/ACCase activities. PCCase and ACCase activities were determined in the presence of the indicated concentrations of haloxyfop. The source of the enzyme was a 4-16% PEG precipitated fraction (A) or the purified PCCase/ACCase preparation (B).
Figure 6. Lineweaver-Burk analysis of the inhibition of maize PCase (A) and ACCase (B) activities by haloxyfop. PCase and ACCase activities were determined as described in Materials and Methods, in the presence of the indicated concentrations of propionyl-CoA and acetyl-CoA, respectively, and with the addition of the indicated concentrations of haloxyfop. The inset present secondary plots of slopes and y-intercepts of the Lineweaver-Burk plots versus haloxyfop concentration. Source of the enzyme was the purified PCase/ACCase preparation.
### Table II. Inhibitors of PCCase and ACCase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition mechanism</th>
<th>$K_i$ (μM)$^a$</th>
<th>PCCase</th>
<th>ACCase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloxyfop</td>
<td>Mixed</td>
<td>$K_i = 0.18 \pm 0.02$</td>
<td>$K_i' = 0.49 \pm 0.07$</td>
<td>$K_i = 0.37 \pm 0.06$</td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>Competitive</td>
<td>14.6±2.3</td>
<td></td>
<td>23.1±2.1</td>
</tr>
<tr>
<td>Methylmalonyl-CoA</td>
<td>Competitive</td>
<td>39.2±4.2</td>
<td></td>
<td>57.7±5.6</td>
</tr>
</tbody>
</table>

$^a$Ki values are the average of two independent experiments.
Figure 7. Chemical structure of haloxyfop and propionyl-CoA. The similarity between the structures is indicated by the bold typeface.
Both malonyl-CoA and methylmalonyl-CoA acted as inhibitors of the PCCase and ACCase reactions assayed with the purified enzyme preparation. In both instances, inhibition was competitive relative to the acyl-CoA substrate (Figures 8 and 9). Inhibition constants were calculated from secondary plots of the Lineweaver-Burk analyses. These determinations revealed that both malonyl-CoA and methylmalonyl-CoA were more potent inhibitors of the carboxylation of propionyl-CoA than the carboxylation of acetyl-CoA (Table II); for both inhibitors, the $K_i$ value for propionyl-CoA carboxylation was approximately $2/3$ of the $K_i$ value for acetyl-CoA carboxylation.

**Substrate specificity.** The purified PCCase/ACCase preparation was used to determine the affinity of the enzyme(s) for the substrates propionyl-CoA and acetyl-CoA. At saturating concentration of the other two substrates (ATP and HCO$_3^-$), the $K_m$ for propionyl-CoA (29.0 μM) was 2-fold lower than the $K_m$ for acetyl-CoA (61.7 μM) (Figures 10A and 10B). However, the maximal rate of carboxylation of propionyl-CoA was less efficient, occurring at approximately 40% that of acetyl-CoA. Thus, although the rate of carboxylation of propionyl-CoA is lower, the affinity for this substrate appears to be higher than that for acetyl-CoA. Similar characteristics have been observed for ACCase from a variety of species. The $K_m$ values for propionyl-CoA are lower or essentially the same to those for acetyl-CoA, for ACCase from *Candida lipolytica* (acetyl-CoA = 0.26 mM; propionyl-CoA = 0.26 mM) (Mishina et al., 1981), rat mammary glands (acetyl-CoA = 50 μM; propionyl-CoA = 45 μM) (Miller and Levy, 1975), and chicken liver (acetyl-CoA = 4.7 μM; propionyl-CoA = 8.8 μM) (Numa, 1969). For all of these ACCases, the rate of carboxylation of propionyl-CoA was between 13% to 20% of that of acetyl-CoA.
Figure 8. Lineweaver-Burk analysis of the inhibition of maize PCCase (A) and ACCase (B) activities by malonyl-CoA. PCCase and ACCase activities were determined as described in Materials and Methods, in the presence of the indicated concentrations of propionyl-CoA and acetyl-CoA, respectively, and with the addition of the indicated concentrations of malonyl-CoA. The insets present the secondary plots of the slopes of the Lineweaver-Burk plots versus malonyl-CoA concentration. Source of the enzyme was the purified PCCase/ACCase preparation.
Figure 9. Lineweaver-Burk analysis of the inhibition of maize PCCase (A) and ACCase (B) activities by methylmalonyl-CoA (MM-CoA).

ACCase and PCCase activities were determined as described in Materials and Methods, in the presence of the indicated concentrations of propionyl-CoA and acetyl-CoA, respectively, and with the addition of the indicated concentrations of methylmalonyl-CoA. The insets present the secondary plots of the slopes of the Lineweaver-Burk plots versus MM-CoA concentration. Source of the enzyme was the purified PCCase/ ACCase preparation.
Figure 10. Lineweaver-Burk analysis of the dependence of maize PCCase/ACCase on acetyl-CoA (a) and propionyl-CoA (b). The purified PCCase/ACCase preparation was assayed as described in Materials and Methods, but with variable concentration of acetyl-CoA or propionyl-CoA.
Potential metabolic functions for PCCase in plants are unclear. Methylmalonyl-CoA formed by the carboxylation of propionyl-CoA could be incorporated into the growing fatty acyl chain during the biosynthesis of fatty acids, giving rise to methyl branched-chain fatty acids (Buckner and Kolattkudy, 1976). Although some plants do synthesize such branched fatty acids, they occur at trace levels, and predominantly occur in the cuticle of the plant (Tulloch, 1976). Furthermore, most of these fatty acids are iso- and anteiso- isoforms, which are thought to arise from the α-keto acids derived from the deamination of valine and isoleucine (O'Hagan, 1991). In the few examples where middle of the chain methyl branching occurs, the methyl group is thought to have arisen by methylation of the straight-chain fatty acid, with the methyl group coming from S-adenosyl methionine (O'Hagan, 1991).

Another potential metabolic function of PCCase is in the oxidation of propionate generated by the catabolism of branched-chain or odd-numbered fatty acids, and the amino acids valine, isoleucine and methionine (Moss and Lane, 1971). In animals, the propionyl-CoA generated by these processes is converted to succinoyl-CoA via the sequential action of PCCase, methylmalonyl-CoA racemase and methylmalonyl-CoA mutase (Voet and Voet, 1990). This process is thought not to operate in plants since methylmalonyl-CoA mutase is a vit B12-dependent enzyme, and this molecule is considered to be absent from plants (Halarnkar, et al. 1988). In fact, Giovanelli and Stumpf (1958) showed that plants metabolize propionate by a "modified β-oxidation" pathway to form acetyl-CoA and CO₂.

To ascertain the significance of PCCase in plant metabolism and determine structure of the enzyme we purified it to near homogeneity, monitoring the purification via the increase in PCCase specific activity. The purification scheme outlined resulted in a preparation that showed a nearly 200-fold higher PCCase specific activity than in the initial
extract. However, throughout this purification scheme, ACCase copurified with PCCase. Indeed, these two activities eluted coincidentaly during fractionation on three separate chromatographic media. In every instance PCCase activity was approximately one third to one half of ACCase activity. In addition, examination of the purified PCCase preparation by SDS-PAGE revealed the presence of two biotin-containing polypeptides of about 220 kDa and 200 kDa, both of which have been shown to be subunits of ACCase (Egli et al., 1993).

These findings strongly suggest that the PCCase activity found in the maize extracts is a side reaction of the ACCase enzyme. Indeed, all ACCases that have been examined are capable of catalyzing, although rather poorly, the carboxylation of propionyl-CoA.

To rule out the possibility that the purified PCCase/ACCase preparation may contain a distinct PCCase enzyme that was undetected by the SDS-PAGE and western analyses done on the preparation, we undertook a number of kinetic characterizations of these two activities using the purified enzyme preparation. The mixed substrate experiments clearly indicate that the carboxylation of propionyl-CoA and acetyl-CoA are catalyzed by a single enzyme in the purified PCCase/ACCase preparation. Consistent with this finding both reactions were similarly inhibited by the monocot-specific herbicide haloxyfop. In addition, both activities were competitively inhibited by the carboxylated products of both reactions, malonyl-CoA and methylmalonyl-CoA.

Finally, we determined the substrate specificity of the purified ACCase by determining the $K_m$ and $V_{rel}$ for the two substrates, acetyl-CoA and propionyl-CoA. These analyses revealed that the maize ACCase has a lower $K_m$ for propionyl-CoA than acetyl-CoA, but the maximal activity obtained with propionyl-CoA is lower than that obtained with acetyl-CoA. Thus, the addition of a methylene group to the substrate does not appear to reduce the binding of the substrate to the enzyme, indeed binding is enhanced. However, the additional methylene group affects catalysis.
The finding that all the PCCase activity in extracts of maize is due to a side reaction of ACCase, alleviates the necessity of explaining the metabolic functions of PCCase. However, it is interesting to contemplate whether the carboxylation of propionyl-CoA, as a side reaction of ACCase, is physiologically significant. This is unlikely to be the case; ACCase occurs in the chloroplasts (Nikolau and Hawke, 1984), and if the catabolic processes that generate propionyl-CoA occur in the mitochondria, as they occur in animals, ACCase is unlikely to ever be presented with propionyl-CoA as a substrate. Thus, at least in maize, the major, if not sole, mechanism for the oxidation of propionate appears to be via the "modified β-oxidation" mechanism originally proposed by Giovanelli and Stumpf (1958).

References


DISCOVERY, PARTIAL PURIFICATION AND CHARACTERIZATION OF GERANOYL-CoA CARBOXYLASE IN Zea mays

A paper to be submitted to Journal of Biological Chemistry
Tomás A. Diez, Eve S. Wurtele and Basil J. Nikolau

Abstract

Geranoyl-CoA carboxylase, a novel plant biotin-containing enzyme, has been partially purified and characterized from maize leaves. Fractionation of the geranoyl-CoA carboxylase activity by hydrophobic interaction chromatography on Propyl-Agarose, affinity chromatography on Cibacron Blue 3GA, and ion exchange chromatography on Q-Sepharose, resolved the geranoyl-CoA carboxylase from other biotin-containing enzymes. The resulting preparation of geranoyl-CoA carboxylase had a specific activity of 138 nmol/min.mg, representing a 180-fold purification. The enzyme has a biotin-containing subunit of 122 kDa. The pH optimum for activity is 8.3. The apparent $K_m$ values for the substrates geranoyl-CoA, bicarbonate and ATP are $64 \pm 5 \mu M$, $0.58 \pm 0.04 \text{ mM}$, and $8.4 \pm 0.4 \mu M$, respectively. Geranoyl-CoA carboxylase occurs ubiquitously through the maize plant. This is the first report of the presence of this enzyme in eukaryotic organisms. A potential metabolic function of geranoyl-CoA carboxylase in the catabolism of monoterpenes is discussed.
Introduction

Geranoyl-CoA carboxylase (GCCase) is a biotin-containing enzyme involved in the catabolism of isoprenoid compounds such as geraniol, citronellol and farnesol (Seubert, 1960; Seubert and Remberger, 1963; Seubert et al., 1963). This enzyme catalyzes the ATP-dependent carboxylation of Z-geranoyl-CoA to form isohexenylglutaryl-CoA (Scheme I) (Seubert and Remberger, 1963). This enzyme has been reported in a few prokaryotic microorganisms including *Pseudomonas citronellolis* (where GCCase was originally discovered by Seubert, 1963), *P. aeruginosa*, *P. mendocina*, and *Acinetobacter* (Cantwell et al., 1978). However, GCCase has been purified only from *P. citronellolis* (Seubert and Remberger, 1963; Fall and Hector, 1977). In this organism, the enzyme is induced by growing the cells with an isoprenoid as sole carbon source.

*P. citronellolis* GCCase is composed of two nonidentical subunits, a biotin-containing subunit of 75 kDa, and a nonbiotin-containing subunit of 63 kDa (Fall and Hector, 1977). The native molecular weight of the holoenzyme is estimated at 520,000-580,000 (Fall and Hector, 1977). Apparently, this enzyme has an A_4B_4 quaternary structure (Fall and Hector, 1977).

Although a great deal is known about the biosynthesis of isoprenoids compounds, almost nothing is known about the way in which these compounds are catabolized. We hypothesized that isoprenoids compounds are catabolized by plants after accomplishing their biochemical functions. One of the enzymes that might be involved in the catabolism of plant isoprenoids is GCCase. Therefore, the search for GCCase in plants was undertaken. To our best knowledge, the presence of this enzyme in eukaryotic organisms has not yet been reported. This paper reports the isolation, purification and partial characterization of GCCase from maize leaves.
Scheme I. ATP-dependent carboxylation of geranyl-CoA by geranyl-CoA carboxylase
Materials and Methods

Plant material. Maize plants were grown in a greenhouse at a constant temperature of 30°C with a daylength, supplemented with artificial lighting, of 14 h/day. Leaves were harvested 2 weeks after planting and immediately frozen in liquid nitrogen.

Radioisotopes and chemicals. The sources of most of radiochemicals and biochemicals have been previously reported (Diez et al., 1994). Citral and ethylchloroformate were obtained from Aldrich. Streptavidin and Protein A were radioactively labeled with carrier-free Na\(^{125}\)I, as described previously (Nikolau et al., 1985), to specific radioactivities of 2.8x10^7 dpm/μg of streptavidin and 1.9x10^7 dpm/μg of Protein A.

Synthesis of geranoyl-CoA. All the reactions were carried out at room temperature. Geranoic acid was prepared by oxidation of citral with Ag\(_2\)O as described by Shamma and Rodriguez (1968). Geranoyl-CoA was synthesized by the mixed anhydride method of Stadman (1957), with slight variations. Briefly, 770 μmoles of geranoic acid were dissolved in 5.1 ml of tetrahydrofuran and neutralized with an equimolar amount of triethylamine. Ethylchloroformate (770 μmoles) was added and the mixture was allowed to stand for 30 min. The mixture was then filtered through glasswool and the anhydride product was recovered in the filtrate. The mixed anhydride compound was added dropwise, over a period of 15 min, to Coenzyme-A (29 μmoles) dissolved in 12 ml of H\(_2\)O:tetrahydrofuran 3:2 (v/v), which had previously been neutralized to pH 8.0 with solid NaHCO\(_3\). The mixture was gently stirred, and to avoid phase separation a few ml of H\(_2\)O were added dropwise. After 20-25 min, the pH of the mixture was adjusted to 3.0 with 2N HCl and the unreactive material was eliminated by 3 extractions with equal volumes of ethyl ether. Finally, the remaining tetrahydrofuran was eliminated by evaporation under vacuum and geranoyl-CoA was recovered by lyophilization. Routinely, 12-15% of the CoA was recovered as geranoyl-CoA.
**Determination of geranoyl-CoA.** The lyophilized geranoyl-CoA was dissolved in 1 mM MES and its concentration was determined by the hydroxamate method of Lipmann and Tuttle (1945).

**Extraction of GCCase.** Maize leaves, frozen in liquid nitrogen, were pulverized with a mortar and pestle. For small scale extraction, 1-3 g of maize leaves were homogenized with 3 volumes of extraction buffer (Diez et al., 1994). An aliquot (0.5 ml) of the extract was passed through a Sephadex G-25 gel filtration column (bed volume = 2 ml) to remove low molecular weight DNA.

Large scale extractions (about 200-250 g) were achieved with 3 volumes of buffer using a Waring blender (Diez et al., 1994).

**Purification of GCCase.** The purification of GCCase was performed at 4°C, from 250 g of maize leaves.

Step 1. PEG precipitation. GCCase was collected from the crude extract by precipitation with powdered PEG 8000 at a final concentration of 18 g/100 ml. The mixture was slowly stirred until the PEG was completely dissolved. The suspension was then centrifuged at 12,200 g for 25 min. The pellet was collected and immediately dissolved in 50-75 ml of buffer A (PEG fraction).

Step 2. Hydrophobic interaction chromatography on Propyl-Agarose. The PEG fraction was applied to a Propyl-Agarose column (1.6 x 20 cm), previously equilibrated with buffer A, which consisted of Hepes 10 mM (pH 7.0), 10-20 mM 2-mercaptoethanol, 1 mM EDTA, and 20% glycerol (v/v). The column was then washed with 200 ml of buffer A. Elution of GCCase was carried out with an 800-ml linear gradient of 0 M to 0.5 M KCl in buffer A. The fractions containing GCCase were pooled and frozen in liquid nitrogen.

Step 3. Affinity chromatography on Cibacron-Blue 3GA Agarose. The pooled fractions from the Propyl-Agarose column were applied to a column of Cibacron-Blue 3GA Agarose (1.6 x 10 cm) previously equilibrated with buffer A. The unbound proteins were
removed by washing the column with 200 ml of buffer A containing 0.075 M KCl. GCCase was then eluted with an 800-ml linear gradient of 0.075 M to 0.75 M KCl in buffer A. Fractions containing GCCase were pooled (Cibacron-Blue fraction).

Step 4. Ion exchange chromatography on Q-Sepharose. The Cibacron-Blue fraction was dialyzed against 8 l of buffer A for 4-5 hours. The dialyzed Cibacron-Blue fraction was then applied to a Q-Sepharose column (2.6 x 18 cm) previously equilibrated with buffer A. The column was washed with 100 ml of buffer A and elution of GCCase was achieved with an 800-ml linear gradient of 0 M to 0.5 M KCl in buffer A. Fractions containing GCCase were frozen in liquid nitrogen and kept separately at -80°C.

Enzyme assay. GCCase activity was determined as the rate of geranoyl-CoA-dependent incorporation of radioactivity from NaH$^{14}$CO$_3$ into the acid stable product, using a procedure based on the method described by Wurtele and Nikolau (1990). Assay conditions are described elsewhere (Diez et al., 1994). Assays were done, at least, in duplicate and the reported kinetic constants are the average of two independent experiments. Lineweaver-Burk analyses of the kinetic data were carried out by using the computer program KaleidaGraph.

Electrophoresis and western blot analysis. The enzyme preparations were analyzed by polyacrylamide gel electrophoresis in denaturing conditions. In doing so, protein samples were adjusted to 50 mM Tris-HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM dithiothreitol, and the samples were placed in a boiling bath of water for 5 min. Proteins were fractionated by electrophoresis in polyacrylamide slabs (16 cm x 18 cm) composed of 5% (w/v) acrylamide/0.26% (w/v) bisacrylamide and containing 0.1% SDS (Laemmli, 1970). After electrophoresis, the gels were stained with Coomassie Brilliant Blue, or subjected to western blot analysis (Kyhse-Andersen, 1984). Biotin-containing polypeptides were detected by incubating the blots with $^{125}$I-streptavidin (Nikolau et al., 1985).
Protein determination. The protein content was routinely determined by the micro-method of Bradford (1976).

**Results and Discussion**

**G GCCase as a novel biotin-containing enzyme in plants.** Initial experiments were undertaken to ascertain if GCCase activity could be detected in extracts of maize leaves. As shown in Table I, an activity consistent with the presence of GCCase was indeed found in extracts from maize leaves. This activity showed an absolute requirement for ATP, Mg$^{2+}$ and geranoyl-CoA for the conversion of radioactivity from NaHCO$_3$ into an acid-stable product. This carboxylation activity is biotin-dependent since it was completely inhibited by avidin; the inhibition by avidin was prevented by preincubating avidin with biotin (Table I). We have observed a similar GCCase activity in extracts from leaves of *Nicotiana tabaccum* and *Arabidopsis thaliana* (unpublished results). These results clearly demonstrate that GCCase is a novel biotin-containing enzyme present in the plant kingdom.

**Tissue distribution of GCCase.** GCCase is widely distributed in the maize plant (Table II). A 2 week old maize seedling was dissected into various organs and GCCase activity determined in extracts from each isolated organ. GCCase activity was fairly constant in the first and second leaves of the seedling and in the roots. However, in the coleoptile GCCase activity was between 1/3 and 1/2 of that found in leaves and roots. These results indicate that GCCase is widely and evenly distributed through the plant.

**Purification of GCCase.** A purification procedure for the purification of GCCase from extracts of maize leaves was developed. The results of the purification of GCCase are illustrated in Figure 1 and summarized in Table III. The enzyme was concentrated from the crude extract by PEG precipitation and nearly 100% of the activity was recovered in the fraction precipitated by 18% PEG. Fractionation of the PEG fraction by hydrophobic
Table I. Incorporation of Radioactivity into an Acid-Stable product from NaHCO$_3$ by Extracts from Maize Leaves.$^a$

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>dpm of $^{14}$CO$_2$ fixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>14033</td>
</tr>
<tr>
<td>-Geranyl-CoA</td>
<td>566</td>
</tr>
<tr>
<td>-ATP</td>
<td>483</td>
</tr>
<tr>
<td>-$^{2+}$Mg</td>
<td>1397</td>
</tr>
<tr>
<td>+Avidin</td>
<td>2228</td>
</tr>
<tr>
<td>+(Avidin + Biotin)</td>
<td>14511</td>
</tr>
</tbody>
</table>

$^a$The complete reaction mixture is described in Materials and Methods.
Table II. GCCase activity in different organs of maize.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Organ</th>
<th>Activity</th>
<th>nmol/min-mg protein</th>
<th>nmol/min-g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots</td>
<td></td>
<td>1.00 ± 0.32</td>
<td>1.89 ± 0.60</td>
</tr>
<tr>
<td>First leaf apical</td>
<td></td>
<td>0.85 ± 0.08</td>
<td>6.57 ± 0.63</td>
</tr>
<tr>
<td>First leaf base</td>
<td></td>
<td>0.61 ± 0.06</td>
<td>1.86 ± 0.18</td>
</tr>
<tr>
<td>Second leaf apical</td>
<td></td>
<td>0.84 ± 0.26</td>
<td>7.8 ± 2.5</td>
</tr>
<tr>
<td>Second leaf base</td>
<td></td>
<td>1.02 ± 0.28</td>
<td>9.5 ± 2.6</td>
</tr>
<tr>
<td>Coleoptile</td>
<td></td>
<td>0.30 ± 0.02</td>
<td>0.78 ± 0.06</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Each value is the average of four determinations from two independent experiments.
Table III. Purification of GCCase from maize leaves*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (nmol/min)</th>
<th>% Recovery</th>
<th>Specific Activity (nmol/min-mg)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>600</td>
<td>720.00</td>
<td>540.0</td>
<td>100.0</td>
<td>0.75</td>
<td>1.0</td>
</tr>
<tr>
<td>18% PEG</td>
<td>100</td>
<td>530.00</td>
<td>533.0</td>
<td>98.8</td>
<td>1.01</td>
<td>1.3</td>
</tr>
<tr>
<td>Propyl-Agarose</td>
<td>63</td>
<td>49.10</td>
<td>463.0</td>
<td>85.7</td>
<td>9.42</td>
<td>12.6</td>
</tr>
<tr>
<td>Cibacron-Blue</td>
<td>180</td>
<td>7.70</td>
<td>196.0</td>
<td>36.3</td>
<td>25.35</td>
<td>33.8</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>9</td>
<td>0.15</td>
<td>21.2</td>
<td>3.9</td>
<td>138.20</td>
<td>184.3</td>
</tr>
</tbody>
</table>

*250 g of plant tissue
interaction chromatography on Propyl-Agarose resulted in a GCCase preparation which contained MCCase activity (Figure 1A). Indeed the elution profile of both these activities were similar, which raised the possibility that these two activities are catalyzed by the same enzyme. This issue was resolved by additional purification of GCCase recovered from the Propyl-Agarose column. Figure 1B shows the elution of GCCase and MCCase activities from the Cibacron Blue affinity matrix. Clearly, GCCase and MCCase are resolved, indicating that they are distinct enzymes.

Finally, to obtain GCCase, free of MCCase, the Cibacron Blue fractions that contained GCCase, but lacked MCCase, were pooled and the sample was subjected to ion exchange chromatography on Q-Sepharose. GCCase eluted from the column at a salt concentration of 0.2 M KCl, with a peak specific activity of 138 nmol/min.mg protein. Thus, GCCase was purified approximately 180-fold, with a recovery of 4%.

Unfortunately, the recovery of protein in the fractions containing GCCase was so low that the subunit(s) of this enzyme could not be detected on Coomassie or silver staining of gels in which proteins had been fractionated by electrophoresis. However, SDS-PAGE followed by western blot analysis with $^{125}$I-streptavidin revealed that the Q-Sepharose fraction, containing peak GCCase activity, contained a single biotin-containing polypeptide with a molecular weight of 122,000. Indeed, similar analysis of aliquots taken from each of the purification steps revealed that this 122,000 Da biotin-containing polypeptide is enriched as GCCase is purified (Fig. 2). Thus, we conclude that the maize GCCase has an 122 kDa biotin-containing subunit.

**Kinetic properties of GCCase.**

**pH optimum.** The activity of maize leaf GCCase was measured in the range between pH 6.3 and 10.3, using Bis-Tris-Propane as the buffer. The enzyme was active over a broad pH range (Fig. 3), with maximum activity occurring at pH 8.3. The enzyme showed 50% of peak activity at pH 7.2 and pH 9.6.
Figure 1. Elution of GCCase Activity on chromatography on Propyl-Agarose (A), Cibacron-Blue 3GA-Agarose (B), and Q-Sepharose (C). See Materials and Methods for details.
Figure 2. Biotin-containing polypeptides at each step of the purification of GCCase from maize leaves.
Alliquots from each fraction of the GCCase purification scheme, outlined in the Materials and Methods, were subjected to SDS-PAGE and western analysis with 125I-streptavidin. A single biotin-containing polypeptide of 122 ± 5 kDa is enriched as GCCase activity is purified. Lane 1, the crude extract; lane 2, the PEG fraction; lane 3, the propyl-agarose fraction; lane 4, the Cibacron Blue fraction; lane 5, the Q-Sepharose fraction.
Figure 3. Effect of pH on GCCase activity. GCCase was assayed as described under Materials and Methods with the exception that the assays were buffered at the indicated pH using 50 mM Bis-Tris-propane.
Kinetic constants. The kinetic parameters, $K_m$ and $V_{max}$, were determined for each of the substrates of GCCase by measuring the activity of the enzyme at varying concentrations of one substrate, while keeping the concentration of the other two substrates at constant and saturating levels. Increasing concentration of each substrate resulted in a hyperbolic increase in GCCase activity, indicating that maize leaf GCCase follows classical Michaelis-Menten kinetics (Fig. 4). The $K_m$ and $V_{max}$ for each substrate was determined from Lineweaver-Burk plots of the data (Fig. 4) and the values of each constant are presented in Table III.

Possible metabolic function of GCCase in plants. Whereas the metabolic function of GCCase in the catabolism of isoprenoids in bacteria has been well established (Seubert and Remberger, 1963; Seubert et al., 1963; Cantwell et al., 1978) (Figure 5), its role in plants is unclear. However, the possible metabolic function of this enzyme in plants could be extrapolated from its function in bacteria. Isoprenoid compounds are actively synthesized by plants to accomplish important physiological functions. In this regard, isoprenoids are found in plants acting as plant growth regulators (such as abscisic acid and gibberellins), part of biologically active molecules such as chlorophylls and prenylquinones, side chains of mitochondrial electron transfer chain components, intermediates of the biosynthesis of polysaccharides and oligosaccharaides, components of biological membranes, attractants for insect pollination, and as defense molecules against fungal and bacterial infections (Callow, 1987). We speculate that the breakdown of molecules bigger than geranoic acid (such as sequiterpenes, diterpenes, etc.), will generate geranoic acid-like monoterpenes which might be further catabolized through the pathway shown in Figure 5. This proposed pathway will enable plants to recycle carbon by forming acetyl-CoA. The action of GCCase in this process is to enable the removal of the methyl branching of the isoprenoid by $\beta$-oxidation. The resulting $\beta$-ketoacid product could subsequently undergo two additional $\beta$-oxidation
Figure 4. Lineweaver-Burk analysis of the dependence of maize GCCase on geranyl-CoA (A), ATP (B), and bicarbonate (C). The purified GCCase preparation was assayed as described in Materials and Methods, but with variable concentration of geranyl-CoA, ATP, and bicarbonate.
Figure 5. Potential metabolic function of maize leaf GCCase as extrapolated from *Pseudomonas citronellolis*. This metabolic pathway is modified after Cantwell et al., 1978. A and B represent uncertain reactions in the pathway. 1= Hydratase; 2= Lyase.
cycles to generate 3-methylcrotonyl-CoA. The catabolism of this compound could be achieved with the aid of an additional biotin-containing enzyme, 3-methylcrotonyl-CoA carboxylase, which has recently been characterized from plants (Diez, et al., 1994). Thus, we predict that the catabolism of monoterpenes in plants utilizes two novel biotin-containing enzymes, making the catabolism of these compounds possible via β-oxidation.

In summary, GCCase represents the second novel biotin-containing enzyme to be discovered in plants recently. This is the first report of this enzyme in a eukaryotic organism. The characteristics of the enzyme that we have described indicate that the plant GCCase may be distinct from the bacterial enzyme.

References

GENERAL SUMMARY

This thesis presents the purification and biochemical characterization of three biotin-containing carboxylases from plants: MCCase, PCase, and GCCase.

MCCase has been purified to near homogeneity from maize leaves. The enzyme is composed of two nonidentical subunits: a biotin-containing subunit of 80 kDa, and a nonbiotin-containing subunit of 58 kDa. The native molecular weight of the enzyme is estimated at 853,000. The holoenzyme probably has an αβ6 configuration. The kinetic constants for the substrates of the enzyme, 3-methylcrotonyl-CoA, ATP, and bicarbonate, and its pH optimum have been determined. MCCase was found to be allosterically activated by Mg^{2+} and is also nonessentially activated by by monovalent cations. The enzyme is inhibited by sulfhydryl and arginyl modifying reagents, suggesting that these residues are involved in catalysis. The fact that MCCase is strongly inhibited by acetoacetyl-CoA suggests that the enzyme may be regulated by feedback inhibition since acetoacetate is one of the final product of the metabolic pathway in which MCCase is involved.

The effect of K^+ on MCCase has been kinetically studied. K^+ increases the affinity of the enzyme for ATP and has no effect on the binding of bicarbonate. However, K^+ and 3-methylcrotonyl-CoA are antagonists since they both mutually increase their dissociation constants for the complex with the enzyme. These results indicate that K^+ is involved in the first half-reaction in which the carboxybiotinyl intermediate is formed.

The kinetic mechanism of soybean MCCase has been determined. Initial velocities studies with competitive inhibitors are consistent with MCCase following a random Bi Bi Uni Uni Ping Pong mechanism in which bicarbonate and ATP bind to the enzyme in a random fashion. An initial rate equation for this mechanism and the values for the kinetic constants were deduced.
A PCCase activity present in maize leaves has been purified. This activity co-purified with ACCase. The preparation contained two biotinylated polypeptides of about 200 and 220 kDa. These two biotin-containing polypeptides have been previously identified as components of ACCase. Inhibition studies with specific inhibitors of ACCase (malonyl-CoA, methylmalonyl-CoA, and haloxyfop) and mixed substrate analyses revealed that the carboxylation of propionyl-CoA is a side reaction of ACCase. These results suggest that maize leaves do not contain a specific PCCase and that propionate is probably metabolized through the modified β-oxidation pathway.

GCCase has been discovered in the plant kingdom. The enzyme contains a biotin-containing subunit of 122 kDa. The kinetic constants for its substrates, geranyl-CoA, ATP and bicarbonate, and its pH optimum have been determined. A potential metabolic pathway for the recycling of carbon from the catabolism of terpene compounds in which GCCase and MCCase may be involved is proposed. Further studies on the structure, metabolic function and regulation of plant GCCase are required.

The characterization of maize leaf MCCase, the absence of PCCase in maize leaves, and the discovery of a novel biotin-containing enzyme in plants, GCCase, have provided a better understanding about the nature of biotin-containing enzymes in plants.
ACKNOWLEDGMENTS

My first expression of sincere gratitude is for my major professor, Dr. Basil J. Nikolau, who, as a friend and teacher, conducted me through the fascinating world of plant biochemistry. His scientific guidance and suggestions will be part of my professional life. I would like also to thank him for his help in the preparation of this thesis.

I am also very thankful to Dr. Donald Graves, Dr. Donald Beitz, Dr. Eve Wurtele, and Dr. Martin Spalding for being part of my committee, and for their advice.

I would like to thank Dr. Herbert Fromm for his expert advice and helpful guidance in enzyme kinetics.

I would also like to acknowledge Dr. Jianping Song and Mr. Tuan-Nam Wen for sharing their hard work and knowledge during the development of the kinetic studies.

I am also grateful to Dr. Robert Thornburg for substituting for Dr. Beitz in my final examination.

I am also thankful to all my partners in Dr. Nikolau's lab for their friendship, encouragement, and support during my doctoral studies.

I would like also to acknowledge LASPAU, which made possible my doctoral studies in the United States. I am very thankful to my scholar advisor, Sonia Wallenberg.

I am especially grateful to my family, Telvia's family, and my friends for their extensive support.