

Minireview

Plant biotin-containing carboxylases[☆]

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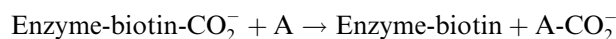
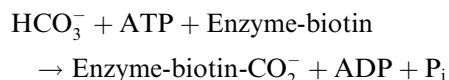
Abstract

Biotin-containing proteins are found in all forms of life, and they catalyze carboxylation, decarboxylation, or transcarboxylation reactions that are central to metabolism. In plants, five biotin-containing proteins have been characterized. Of these, four are catalysts, namely the two structurally distinct acetyl-CoA carboxylases (heteromeric and homomeric), 3-methylcrotonyl-CoA carboxylase and geranyl-CoA carboxylase. In addition, plants contain a noncatalytic biotin protein that accumulates in seeds and is thought to play a role in storing biotin. Acetyl-CoA carboxylases generate two pools of malonyl-CoA, one in plastids that is the precursor for de novo fatty acid biosynthesis and the other in the cytosol that is the precursor for fatty acid elongation and a large number of secondary metabolites. 3-Methylcrotonyl-CoA carboxylase catalyzes a reaction in the mitochondrial pathway for leucine catabolism. The exact metabolic function of geranyl-CoA carboxylase is as yet unknown, but it may be involved in isoprenoid metabolism. This minireview summarizes the recent developments in our understanding of the structure, regulation, and metabolic functions of these proteins in plants.

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Biotin-containing enzymes catalyze reactions in which a carboxyl group is transferred between substrates. Depending on the chemical nature of the substrate that donates or accepts the carboxyl group, biotin-containing enzymes catalyze carboxylation, decarboxylation, or transcarboxylation reactions [1]. Thus, carboxylases use bicarbonate ion as the carboxyl-group donor and the acceptor is an organic molecule, decarboxylases use an organic molecule as the donor and the carboxyl-group acceptor is water, and transcarboxylases use organic molecules as both the carboxyl-group donor and the acceptor. In these enzymes, the catalytic role of the biotin prosthetic group is to react with the carboxyl group and carry it between the two active subsites of the enzyme, each of which catalyzes a distinct half-reaction of the overall reaction [1,2]. The first of these two half-reactions is the transfer of the carboxyl group from the

donor substrate to the 1'-N atom of the biotin prosthetic group (the biotin carboxylation reaction). The second half-reaction is the transfer of the carboxyl group from carboxy-biotin to the acceptor substrate (the carboxyl-transferase reaction). This article will review the status of research into biotin-containing enzymes from higher plants. Because these enzymes all catalyze carboxylation reactions, we will concentrate on biotin-dependent carboxylases. These enzymes catalyze two-step reactions, where A represents the acceptor substrate that is carboxylated:



Hence, biotin-dependent carboxylases catalyze the formation of a new carbon-carbon bond, and the energy required for the formation of this bond is derived from the hydrolysis of ATP [2]. This energy of hydrolysis is coupled to the reaction by the fact that ATP reacts with the bicarbonate ion to form carboxy-phosphate, which then reacts with biotin to form carboxy-biotin.

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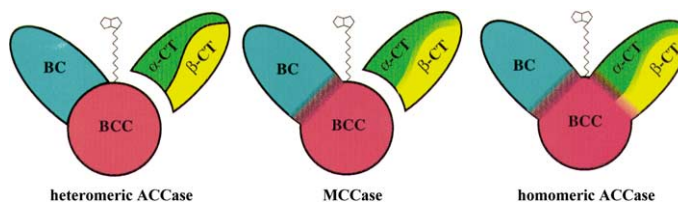


Fig. 1. Schematic representation of the distribution of the BC, BCC, and CT functional domains among different subunits of biotin-dependent carboxylases. Each subunit is represented by a black boundary line. Subunits that are in contact are considered to be in a tight complex, whereas those that are separated by a gap are readily dissociable. The BCC domain carries the biotin prosthetic group, which is thought to swing between the BC and the CT active sites. At the BC active site biotin is carboxylated, and at the CT active site the carboxyl group is transferred to the final product of the reaction.

Biotin-dependent carboxylases have three distinct functional domains (Fig. 1) [3,4]. These domains can be readily recognized based upon a high degree of sequence conservation. Two are catalytic, and the third is a structural domain. The structural domain that carries the biotin prosthetic group is called the biotin carboxyl-carrier (BCC)¹ domain. One of the catalytic domains catalyzes the carboxylation of the biotin prosthetic group and is called the biotin carboxylase (BC) domain. The other catalyzes the transfer of the carboxyl group from carboxy-biotin to the carboxyl-group acceptor substrate and is named the carboxyltransferase (CT) domain. The quaternary structural organization of these three domains (BCC, BC, and CT) differs among different biotin-containing enzymes (Fig. 1) and among the same enzymes from different organisms. Furthermore, each of these domains can be on different protein subunits, such as the heteromeric ACCase of archaea, bacteria, and plastids of dicotyledonous plants. In other enzymes, either two or all three domains can be carried on a single polypeptide. For example, all three domains are on a single polypeptide in the case of the homomeric ACCase from animals [5], yeast [6], diatoms [7], and plant cytosol [8]. This is also the situation for the animal [9] and yeast [10] pyruvate carboxylase and for urea carboxylase [11]. However, in the case of 3-methylcrotonyl-CoA carboxylase [12,13] and propionyl-CoA carboxylase [14], the BC and BCC domains are fused into a single polypeptide and the CT domain is on a separate polypeptide.

Characterization of biotin-containing enzymes in plants has historically been driven by efforts to understand the regulation of *de novo* fatty acid biosynthesis [15], a process that occurs predominantly in plastids of plant cells. Thus, primary interest has focused on the identification of the structure and regulation of ACCase, which catalyzes the ATP-dependent carboxylation of

acetyl-CoA to form malonyl-CoA. This reaction is classically considered to be the first and committing reaction of *de novo* fatty acid biosynthesis, and biochemical characterizations of this enzyme from animals, bacteria, and yeast indicate that it catalyzes a reaction that is important in regulating the flux through fatty acid biosynthesis [16–19]. Until recently, studies of ACCase and other biotin-containing enzymes of plants were complicated by the fact that plants contain two structurally distinct isoforms of ACCase [20–22] and biotin-proteins whose identities were unknown [23,24]. Much of this complexity and apparent inconsistency in the literature has now been resolved with the application of recombinant DNA technology [15]. Specifically, in addition to the two isozymes of ACCase, plants also contain the biotin enzymes 3-methylcrotonyl-CoA carboxylase (MCCase) [25] and geranyl-CoA carboxylase (GCCase) [26] and a seed storage biotin-protein (SBP) [27]. Characterization of each of these biotin-proteins continues to open new avenues of research into metabolic processes that are poorly characterized in plants (Fig. 2).

Heteromeric ACCase

Enzyme structure

Heteromeric ACCase, which occurs in plastids of most non-Graminaeae plants, is composed of four distinct subunits. Two of these subunits (the α - and β -CT subunits) constitute the CT catalytic domain, and the other two subunits constitute the BC and BCC domains of this enzyme. This subunit organization is analogous to the ACCase that was first characterized in bacteria [28] and more recently in some species of archaea [29].

The heteromeric ACCase in plants was first alluded to by the work of Kannangara and Stumpf [30,31]. They showed that stromal extracts from isolated spinach chloroplasts could support the two catalytic half-reactions of ACCase, the ATP-dependent carboxylation of biotin and the transfer of the carboxyl group from carboxybiotin to acetyl-CoA to form malonyl-CoA, and

¹ Abbreviations used: BCC, biotin carboxyl-carrier; BC, biotin carboxylase; CT, carboxyltransferase; ACCase, acetyl-CoA carboxylase; MCCase, 3-methylcrotonyl-CoA carboxylase; GCCase, geranyl-CoA carboxylase; HCS, holocarboxylase synthase; SBP, seed storage biotin-protein.

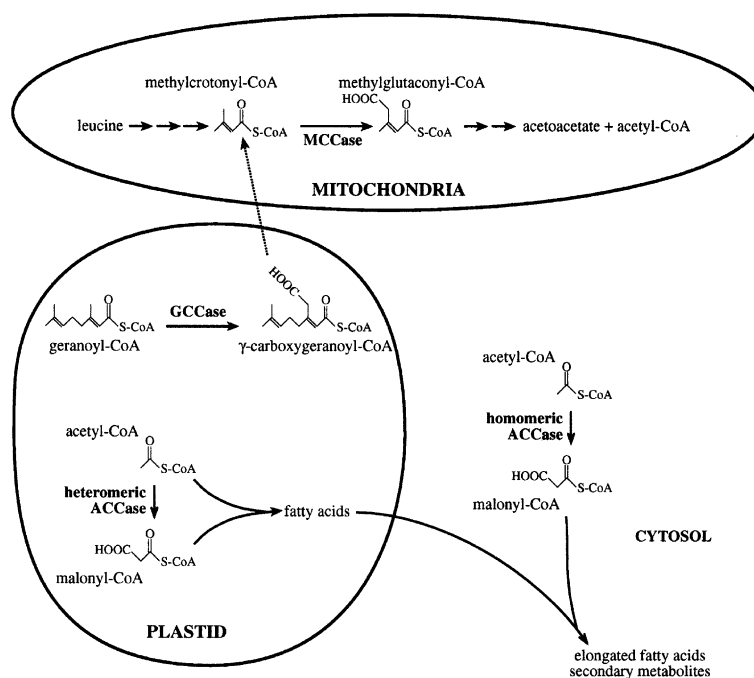


Fig. 2. Metabolic functions of biotin-dependent carboxylases. The heteromeric ACCase in plastids generates the malonyl-CoA that is used as the substrate for de novo fatty acid biosynthesis. The homomeric ACCase in the cytosol generates the malonyl-CoA that is used to elongate fatty acids and for the synthesis of a variety of secondary metabolites. MCCase in mitochondria is required in the catabolism of leucine. In addition, MCCase in conjunction with the plastidic GCCase may also be required in the catabolism of acyclic isoprenoids. In plants of the Graminaeae family, the heteromeric form of ACCase does not occur and is replaced in plastids by the homomeric enzyme.

that the BCC component was associated with the thylakoid membranes. However, this observation was inconsistent with earlier [32–34] and subsequent [35–42] findings of a soluble, nondissociable ACCase in a number of plant species. We now understand that many of the apparent inconsistencies in these reports are the consequence of the fact that in most plants the plastidic isoform of ACCase is a heteromeric enzyme that is unstable due to its dissociation, whereas the homomeric ACCase (present in the cytosol of all plants) is larger, more stable, and nondissociable.

The presence of the heteromeric ACCase in plants began to be confirmed following the sequencing of the *Escherichia coli accD* gene [43] that codes for the β -CT subunit of this enzyme. This led to the homology-based identification of a chloroplastic operon (called *ZfpA* in pea, ORF316 in liverwort, and ORF512 in tobacco) [44], which was subsequently shown to be the β -CT subunit of the plant heteromeric ACCase [45]. Very quickly after this, cDNAs and genes coding for the BCC [46–50], BC [48,51,52], and α -CT subunits [48,53,54] were characterized from a number of plant species.

Biochemical and molecular characterizations of plant heteromeric ACCase have been undertaken for a number of different species, including *Arabidopsis thaliana*, pea, soybean, and *Brassica napus*. Many recent characterizations have been conducted via the cDNA or genomic sequences coding each subunit. For the soybean

subunits, the N-terminal sequences of the mature proteins have been directly determined [48]. In combination with computer algorithms that predict plastid-targeting sequences, it is possible to infer the sequences of the mature subunits for the nuclear-encoded subunits of additional species.

There is considerable conservation in the primary structure of each subunit among different plant species, and each subunit shares lesser but significant sequence similarity with bacterial homologs (typified by the *E. coli* enzyme [28]). In fact, each of the subunits of the plant heteromeric ACCase also shares sequence similarity with analogous domains of other biotin-containing enzymes. For example, the BC subunit of the heteromeric ACCase shares significant sequence similarity with BC domains from almost all biotin-containing enzymes, including pyruvate carboxylase, propionyl-CoA carboxylase, MCCase, urea carboxylase, and most remotely, homomeric ACCase [51]. Of the four heteromeric ACCase subunits, the 50-kDa BC subunit is the most highly conserved among plant species. In contrast, there is considerable sequence polymorphism among BCC subunits from different species. In addition, in all species examined to date, multiple size-variants of the BCC subunit appear to accumulate in the same organ [49,50]. The mature BCC subunits are polypeptides of about 200 residues. The highest sequence conservation among BCC subunits is concentrated at the C-terminal half of the

protein where the highly conserved “(A/I/V)-M-K-(L/V/M/A/T)” biotinylation signature sequence is present. Because these proteins contain an alanine- and proline-rich hinge region, their migration on SDS-PAGE is anomalous, and the apparent molecular weights of the mature proteins are in the range of 22 to 37 kDa [46,48–50]. The α - and β -CT subunits also show considerable sequence and length heterogeneity among different plant species [45,48,53,54]. For example, the α -CT subunit is between 690 and 875 residues. In comparison, the bacterial α -CT subunit (typified by *E. coli*) is only 319 residues in length. The sequence homology among the plant α -CT subunits is concentrated to the region of the polypeptide that is homologous with the bacterial protein, and the C-terminal halves are diverse in sequence and length. This C-terminal tail appears not to be required for catalysis and may in fact have some structural role [55]. Similarly, the β -CT subunits show sequence diversity among plant species, and the plant proteins are almost twice the size of the bacterial homologs.

The quaternary structure of the plant heteromeric ACCase is still to be fully elucidated. Gel filtration chromatography indicates that the holoenzyme may have a molecular mass of about 700 kDa [48,56]. A direct measurement of the molecular mass of this enzyme is very difficult to determine because the enzyme readily dissociates and becomes inactivated during purification. However subcomplexes can be recovered and used to reconstitute the active holoenzyme [48,56]. Such biochemical fractionations have established that, as in *E. coli*, the α -CT and β -CT subunits are in a tight complex that survives vigorous fractionation, coimmunoprecipitation, fractionation by ion exchange and gel filtration chromatography, and electrophoresis [22,48,54,56]. The other subcomplex that is recoverable from the fractionation of the plant heteromeric ACCase contains the BCC and BC subunits [48,56]. In contrast, fractionation of *E. coli* ACCase yields BC and BCC subcomplexes, each of which is a dimer [57–59]. Although plastid ACCase is often recovered as a soluble protein, several studies have reported membrane associations [45,53,60]. Recently, at least 50% of ACCase protein of young pea leaf chloroplasts was found to be tightly associated with the inner envelope membrane [61]. Attachment to the envelope is through the CT subunits and is resistant to salt or detergent, implying that ACCase may interact strongly with other proteins in the membrane or perhaps is anchored in other ways.

Gene organization

Nuclear genes code for three of the four-heteromeric ACCase subunits (BCC, BC, and α -CT). Thus, these subunits are synthesized in the cytosol as larger precursors and subsequently imported into plastids, processed, and assembled with the plastid-coded subunit

(β -CT). With the exception of *Arabidopsis*, all higher plants examined have multiple copies of the nuclear genes that code for these subunits. The occurrence of paralogous genes for each nuclear-coded subunit has been inferred from sequence heterogeneity among cDNAs [48,49] and analysis of expressed sequence tag collections [62]. For example, the soybean [48] and *B. napus* genomes contain multiple copies of the α -CT and BCC subunits [49]. Moreover, each of these paralogous genes codes for a variant of the subunit. If paralogous genes are expressed in the same cell, there is potential for considerable heterogeneity of subunits in the holoenzyme. These holoenzymes could have different biochemical properties. In *Arabidopsis*, single genes code for the BC (At5g35360) [51] and α -CT (At2g38040) [54] subunits, but at least two genes code for the BCC subunit (At5g16390 and At5g15530) [47,50].

Regulation of gene expression

The regulation of the biosynthesis of the heteromeric ACCase has been primarily studied by monitoring the accumulation of the individual subunits and their corresponding mRNAs during plant development. These studies have specifically focused on developing seeds, where fatty acids are used either in the assembly of membrane lipids or for the biogenesis of triacylglycerol that is deposited as oil. Consistent with the metabolic demands on the plastidic malonyl-CoA pool, the ACCase subunits and their mRNAs accumulate to high levels in young developing seeds and leaves [46,47, 49–51,54]. At least for the BC subunit, regulation of the transcription of the BC gene appears to be one of the mechanisms for altering BC subunit expression [63].

In *Arabidopsis*, detailed quantification of the accumulation of the mRNAs of the heteromeric ACCase indicates that four (BCC-1, BC, α -CT, and β -CT) of the five subunit mRNAs accumulate in a coordinated fashion, keeping a constant molar ratio during development [54]. This coordination is also evident in the changes that occur in the spatial distribution of these mRNAs as the siliques, and the seeds within, develop [54]. Furthermore, mRNAs coding for additional functions in fatty acid and lipid biosynthesis (e.g., plastidic pyruvate dehydrogenase, 3-ketoacyl-ACP reductase, enoyl-ACP reductase, acyl-ACP thioesterase, and stearoyl-ACP desaturase) appear to accumulate synchronously, keeping an equal molar ratio during development [64,65]. A more expansive study using DNA microarrays to profile 3500 mRNAs during the development of *Arabidopsis* seeds has identified three temporal expression profiles for the genes that comprise the lipid biosynthetic network [66]. The profile that contains the heteromeric ACCase also contains many other fatty acid biosynthetic genes, including those coding for ACP, 3-ketoacyl-ACP synthase I, and enoyl-ACP reductase.

Homomeric ACCase

Enzyme structure and gene organization

Homomeric ACCase is a 500-kDa enzyme that is composed of two identical subunits. In contrast to the heteromeric ACCase, in which the functional domains of the enzyme are separated on distinct subunits, in the homomeric ACCase these domains are fused into a single polypeptide. The subunits of all homomeric ACCases from animals, fungi, and plants share a common structural organization corresponding to the linear arrangement of the functional domains: NH₂-BC-BCC-CT-COOH. Hence, all homomeric ACCases share a high degree of sequence conservation.

The homomeric ACCase was initially characterized from animals and yeast. In plants, this enzyme was first detected in wheat germ [32,34]. However, the structure of the enzyme was not realized until it was purified with monomeric-avidin affinity chromatography [38–40].

Initially, a homomeric ACCase gene was cloned from the diatom *Cyclotella cryptica* [7]. Soon after, homomeric ACCase genes and cDNAs were characterized from higher plants, including those from alfalfa [8], *Arabidopsis* [67,68], *B. napus* [69], maize [70,71], and wheat [72,73]. In dicots, the absence of a plastid-targeting peptide at the N-terminal end of the homomeric ACCase subunit indicated that it is a cytosolic enzyme.

In all plant species examined to date, a small gene family encodes the homomeric ACCase. With the exception of *Arabidopsis*, whose genome is completely sequenced, the full complement and arrangement of homomeric ACCase genes in plant genomes have not been determined. However, comprehensive work that indicates that some homomeric ACCase genes are clustered in the genome has been conducted in wheat [74–76] and in *B. napus* [77]. Wheat (and presumably other Graminaeae), which lacks the heteromeric ACCase in plastids, has two isoforms of homomeric ACCase, one localized to plastids and the other in the cytosol. In the genome of hexaploid wheat, a single *Acc-1* gene for each group-2 homeologous chromosome is recognizable as coding for a plastid-targeted ACCase [75,76]. The cytosolic homomeric ACCase is encoded by at least five genes (*Acc-2,1*; *Acc-2,2*; *Acc-2,3*; *Acc-2,4*; *Acc-2,5*), which are located at two distinct loci [74].

In the *Arabidopsis* genome, two tandemly repeated genes (*ACC1* and *ACC2*) code for the homomeric ACCase [67,68]. With the exception of the N-terminal end of the *ACC2*, these two genes code for near-identical proteins (over 90% sequence identity) [78]. The N-terminal end of the *ACC2* protein is extended relative to *ACC1*, and this extension appears to be a plastid-targeting transit peptide [78], as occurs with one of the *B. napus* homomeric ACCase isozymes [77]. These characterizations of the structure of the homomeric

ACCcase genes indicate that plastids of dicots (at least *B. napus* and *Arabidopsis*) contain both heteromeric ACCcase and homomeric ACCcase isozymes. Based on lower expression levels of homomeric ACCcase in *B. napus* plastids and their very low propionyl-CoA carboxylase activity [79], the quantitative contribution of this redundant mechanism for generating plastidic malonyl-CoA is likely to be low. Nevertheless, this occurrence would represent a redundancy in the malonyl-CoA-generating mechanism in plastids, which might have allowed for the evolutionary loss of the heteromeric ACCcase from the genome of the Graminaeae [21]. Moreover, a similar evolutionary loss of the heteromeric ACCcase may also be occurring in other plant taxa [80,81].

Regulation of gene expression

Most studies on the expression of homomeric ACCcase have focused on the mechanisms that control the biosynthesis of the enzyme during growth and development or in response to environmental stimuli. In animals and yeast, ACCcase activity is regulated by both the control of gene transcription and the posttranscriptional events [e.g., 82,83]. Because in these organisms the homomeric ACCcase generates the malonyl-CoA precursor for de novo fatty acid biosynthesis, these regulatory mechanisms primarily couple the expression of the homomeric ACCcase genes to the need of this biosynthetic process.

In contrast, the cytosolic homomeric ACCcase of plants generates a malonyl-CoA pool that has a different metabolic fate. In plants, the cytosolic malonyl-CoA pool is an intermediate required for the elongation of fatty acids and the biosynthesis of a variety of secondary metabolites, including flavonoids, stilbenoids, malonic acid, and malonyl-derivatives. Thus, the expression of homomeric ACCcase genes follows varied developmental and metabolic cues [8,78,84,85].

In alfalfa cells, ACCcase could be considered part of the flavonoid biosynthetic pathway, whose expression is induced either by UV radiation [86] or by fungal elicitors [87]. Shorosh et al [8] established that elicitor-induced alterations in ACCcase expression are due to enhanced accumulation of the ACCcase mRNA. This ACCcase mRNA induction is probably part of a large network of genes that coordinately responds to environmental signals to induce flavonoid biosynthesis [84].

In *Arabidopsis*, *ACC1* accounts for the vast majority of homomeric ACCcase expressed. This difference in expression level between *ACC1* and *ACC2* is primarily due to enhanced transcription of the *ACC1* gene. Thus, even though *Arabidopsis* appears to have the genetic capacity to generate redundant malonyl-CoA pools in plastids (one derived via the heteromeric ACCcase and the other

derived via the *ACC2* gene product), this redundancy is avoided as the *ACC2* gene is transcribed at less than 1% of the level of *ACC1* [78]. Furthermore, the homomeric ACCase mRNA accumulates in a developmental pattern that closely correlates with that of ATP-citrate lyase, which is the enzyme that generates the cytosolic pool of acetyl-CoA, the substrate for the homomeric ACCase [85]. Indeed, the spatial and temporal accumulation patterns for these two mRNAs are consistent with the role of these two enzymes in fatty acid elongation [88,89] and the biosynthesis of flavonoids.

Enzymological properties of the heteromeric and homomeric acetyl-CoA carboxylases

Basic properties

In most tissues from dicotyledonous plants the heteromeric ACCase is responsible for the bulk of in vivo flux of acetyl-CoA to malonyl-CoA. However, assays of ACCase have sometimes indicated greater activity of the homomeric form [e.g., 90]. In part this is due to the tendency of subunits of the heteromeric ACCase to dissociate and lose activity during extraction.

Kinetic studies on heteromeric ACCase have indicated K_m values of 100–250 μM for acetyl-CoA, 150–250 μM for Mg-ATP and 0.6–1 mM for HCO_3^- [e.g., 22]. The K_m for acetyl-CoA is severalfold higher than the estimated concentration of acetyl-CoA in chloroplasts [91], suggesting that concentration of this substrate could limit ACCase activity. K_m values for homomeric ACCase are generally lower for acetyl-CoA (5–50 μM) and higher for HCO_3^- [e.g. 22,36,92].

The catalytic, chemical, and reaction mechanisms of ACCase are likely the same for both heteromeric and homomeric enzymes [28]. Early kinetic studies of castor seed and maize ACCase concluded that the reaction mechanism was ping-pong [42] in which the ADP and Pi products of the BC domain are released before binding of acetyl-CoA. More recent analysis of maize leaf ACCase suggests a Ter-Ter mechanism in which a ternary complex forms between ADP, Pi, and acetyl-CoA at the carboxyltransferase [92]. This is analogous to the mechanism proposed for animal and *E. coli* ACCase [28,93]. Because regulation by both redox and phosphorylation occurs at the CT subunits, it is likely that the CT subunit reaction is rate determining for overall ACCase. If this is the case, the biotin prosthetic group of ACCase may primarily exist as carboxy-biotin.

In addition to their structure and K_m values, the heteromeric and homomeric ACCases can be distinguished in two other ways. First, the cytosolic and plastid homomeric enzymes can carboxylate propionyl-CoA at rates approximately 15–50% of that for acetyl-CoA [90,94,95],

but this activity is undetectable with the heteromeric enzyme. This feature makes it possible to distinguish the homomeric enzyme activity in extracts that contain both forms [79]. Indeed, this side reaction of the homomeric ACCase was probably the basis of the reported presence of propionyl-CoA carboxylase in plants [24].

Second, the homomeric enzyme is very sensitive to two classes of economically important herbicides (aryloxyphenoxy propionates and cyclohexanediones) [96–101], and this distinction provides a basis for the selective action of these herbicides. The homomeric enzyme of Graminaeae chloroplasts is particularly sensitive with IC_{50} values in the 10–100 nM range for diclofop. The cytosolic enzymes of both maize and pea are 50 times less sensitive [20,22,92]. Detailed molecular studies of herbicide-sensitive and herbicide-resistant homomeric ACCase identified a specific Ile to Leu substitution in the CT domain of the enzyme that changes the wheat plastid ACCase from sensitive to resistant [102,103]. Indeed, such a mutational change in the homomeric ACCase sequence has been found to confer herbicide resistance in wild oat [104] and green foxtail (*Setaria viridis* L. Beauv.) [105].

Regulation of heteromeric ACCase

In vivo evidence for ACCase having a regulatory role in plant fatty acid biosynthesis was provided by measurements of the pools of acetyl-CoA, malonyl-CoA, acetyl-ACP, malonyl-ACP, and other acyl-ACP intermediates of fatty acid synthesis. Spinach plants [106] and isolated pea and spinach chloroplasts [91] were examined under light and dark conditions that differ by at least 10-fold in rates of fatty acid synthesis. Consistent with the ACCase reaction being far from thermodynamic equilibrium (and therefore, a potential site of regulation), acetyl-CoA pools were much larger than malonyl-CoA pools. Furthermore, after the transition from high rates of fatty acid synthesis in the light to low rates in the dark, malonyl-ACP concentration decreased to undetectable levels while the acetyl-ACP concentration increased. These observations, plus the fact that other acyl-ACP pools do not change significantly in the light–dark transition, indicate that the ACCase reaction is a major determinant of the flux of carbon through the pathway. A similar conclusion was obtained by inhibitor studies of barley and maize ACCase [107]. Both acetate incorporation into fatty acids and activity of homomeric ACCase were strongly inhibited by the herbicides fluazifop and sethoxydim [98]. By comparing concentration curves for inhibition of ACCase in vitro with their effects on ^{14}C -acetate incorporation into fatty acids in vivo, strong apparent flux control coefficients of 0.58 and 0.52 were determined for ACCase of barley and maize leaves, respectively.

Light/dark control of acetyl-CoA carboxylase activity

Fatty acid synthesis in photosynthetic tissues is strongly light dependent [108,109], and the ACCase reaction is likely the major site of this regulation. ACCase from wheat germ, maize leaves, and spinach chloroplasts is inhibited by ADP at concentrations similar to the K_m for ATP [36,110]. Upon illumination, the ATP/ADP ratios, $[Mg^{2+}]$, and pH in the chloroplast stroma all change in directions that increase ACCase activity. Based on analysis of maize leaf ACCase, Nikolau and Hawke [36] calculated that these stromal changes could account for a 24-fold activation in ACCase activity. Spinach chloroplast ACCase activity was also stimulated 10-fold by assay conditions that mimic stromal conditions in the light compared to assays under simulated dark conditions [111].

ACCase activity in desalted leaf extracts can be increased severalfold by sulfhydryl reagents, including dithiothreitol and thioredoxin [112,113], and these results indicate that ACCase is subject to redox regulation similar to that of several key enzymes of photosynthesis. In pea, activation is mediated through reduction of a disulfide bond between the α -CT and the β -CT subunits [55].

Phosphorylation of serine residue(s) of the β -CT subunit occurs in pea chloroplasts incubated in the light [114]. Alkaline phosphatase treatment reduces ACCase activity in parallel to removal of phosphate groups from ACCase. This activation by phosphorylation is opposite to the inhibition of animal ACCase by phosphorylation but is consistent with the increase in ATP concentration and rates of fatty acid synthesis in chloroplasts in the light and the activation of other plastid enzymes by phosphorylation.

After identical extraction, 2- to 3-fold higher ACCase activity is recovered from isolated chloroplasts incubated in the light compared to those incubated in the dark [111,115]. Yet, within 5 min after extraction, ACCase activity from dark-incubated chloroplasts increased, suggesting a transient inactivation or inhibition of ACCase in the dark. Because assay conditions were identical and phosphorylation or redox reduction is unlikely to occur after chloroplast lysis/dilution, this result suggests that other unknown mechanisms may activate ACCase in the light.

It should also be noted that when pea or spinach chloroplast ACCase is assayed under physiological concentrations of substrates and cofactors, enzyme activity is 5- to 10-fold lower than the rates required to sustain known in vivo rates of fatty acid synthesis [111]. This observation implies a major loss of ACCase activity upon cell disruption, which also occurs in *E. coli* due to dissociation of the complex [116]. Alternatively, perhaps substrate channeling [117] leads to higher local concentrations of substrates/cofactors than those cal-

culated from stromal volumes and chloroplast analysis. Regardless, the lower-than-expected in vitro activity emphasizes that our understanding of ACCase activity is lacking, and yet-to-be discovered factors may serve to stabilize or activate ACCase in vivo.

Feedback inhibitors and other effectors of acetyl-CoA carboxylase activity

Feedback regulation of ACCase by the end products of fatty acid synthesis would be a logical mechanism to balance the production of fatty acids with their utilization. Indeed, animal ACCase is strongly inhibited by nanomolar concentrations of long-chain acyl-CoAs [118] and *E. coli* ACCase is inhibited by nanomolar concentrations of C6 to C20 acyl-ACPs [119]. In plants, when exogenous lipids are added to tobacco suspension cells, the rate of fatty acid synthesis decreases. Analysis of changes in the acyl-ACP pools indicates that this down-regulation occurs at the ACCase reaction [120]. Because long-chain acyl-CoA is not thought to be an intermediate of lipid metabolism inside the plastids, the most logical intermediates that might act as feedback inhibitors of ACCase activity are long-chain acyl-ACP and/or free fatty acids. However, the concentrations of these products increase in vivo when fatty acid synthesis is inhibited by exogenous lipids [120] or darkness [91,121]. Furthermore, addition of long-chain acyl-ACP, free fatty acids, or other possible feedback inhibitors (including acyl-CoA) at physiological concentrations to in vitro ACCase assays does not lead to substantial inhibition [56,92,95].

Other effectors that have been investigated for their effect on ACCase activity include CoA and acyl-CoAs, citrate, and monovalent cations, such as K^+ . The heteromeric ACCase is stimulated fourfold by CoA [122], acetyl-CoA, and other short chain acyl-CoAs [111]. Of these, only acetyl-CoA occurs in vivo at concentrations likely to cause stimulation [91]. In contrast, the homomeric ACCase is inhibited by free CoA [36]. Citrate or other tricarboxylic acids that activate animal ACCase have little impact on plant ACCase at physiological concentrations [36,42,123,124]. Finally, monovalent cations, such as potassium, stimulate ACCase activity [36,124] as with other biotin-enzymes such as MCCase [125].

Flux control by acetyl-CoA carboxylase

Although the studies described above clearly indicate that ACCase is a regulatory enzyme in the fatty acid biosynthetic pathway, they do not demonstrate that flux through the pathway can be influenced by manipulation of this enzyme. Regulatory enzymes often fail to influence flux in transgenic experiments because (1) they are subject to feedback or other

regulation that down-regulates the overexpressed enzyme or (2) other steps in the pathway become limiting when one enzyme is overexpressed. Roesler et al. [79] tried to overcome the first limitation by targeting the normally cytosolic homomeric *Arabidopsis* ACCase to plastids of developing *B. napus* seeds. Although the activity of plastid-localized ACCase increased approximately twofold in these experiments, the oil content of the seeds increased by only 5%. Presumably, other steps in the fatty acid biosynthetic pathway became limiting or other downstream factors prevented accumulation of more oil. However, using the same approach, Peter Doermann et al. (Max Plank Institute of Molecular Plant Physiology, Golm) recently found that triacylglycerol content of potato tubers can be increased by severalfold (unpublished observations). In *E. coli*, overexpression of ACCase when combined with a thioesterase to provide a sink for fatty acid products led to a sixfold increase in fatty acid synthesis [126]. Such major differences in flux control by ACCase emphasize that the results of overexpressing an enzyme can be strongly influenced by the genetic background of transgenic organisms.

MCCase

Enzyme structure and gene organization

MCCase (EC 6.4.1.4) catalyzes the ATP-dependent carboxylation of 3-methylcrotonyl-CoA to form 3-methylglutaconyl-CoA. The characterization of MCCase as a biotin-containing enzyme over 40 years ago in bacteria and mammals (reviewed in [1]) led to the identification of the biochemical function of biotin as an enzyme cofactor. In animals and some bacteria, MCCase catalyzes a reaction required in the catabolism of leucine, a pathway of six reactions that degrades leucine to acetyl-CoA and acetoacetate.

As in animals and bacteria, plant MCCase is a heteromeric enzyme composed of two types of subunits: a biotinylated subunit (MCC-A) of about 75–80 kDa and a nonbiotinylated subunit (MCC-B) of about 60 kDa [25]. MCCase has been purified and characterized from a number of plant species. The Michaelis constants (K_m) for the substrates 3-methylcrotonyl-CoA, bicarbonate, and $Mg \cdot ATP$ are in the range of 10–50 μM , 0.8–2 mM, and 20 μM , respectively. The kinetic mechanism of the reaction catalyzed by MCCase is “bi-bi uni-uni ping-pong” (reviewed in [25]). Characterization of the deduced sequences of the MCC-A and MCC-B subunits established that the MCC-A subunit contains BC and BCC functional domains, whereas the MCC-B subunit contains the CT functional domain. In the genome of *Arabidopsis*, a single gene codes for each of the subunits, and these two genes are not linked. The *MCC-A* gene

(At1g03090) is located on chromosome 1, and the *MCC-B* gene (At4g34030) is located on chromosome 4. In other plant species, the MCCase subunits are each encoded by a small gene family (two to four copies per subunit).

Metabolic function and regulation

In addition to its role in leucine catabolism in animals, MCCase is involved in the recycling of carbon from isoprenoids via the mevalonate shunt [127]. In some bacteria that can use isoprenoids as a carbon source, MCCase is thought to have a function in isoprenoid catabolism [128,129]. Until the characterization of MCCase in plants, the catabolism of leucine in these organisms was thought to occur by an MCCase-independent, peroxisomal pathway [130]. However, MCCase is a mitochondrial enzyme [131], and detailed metabolic studies [132] established that plant mitochondria catabolize leucine to acetyl-CoA and acetoacetate, analogous to the pathway found in animals. Thus, plants appear to have two distinct pathways for leucine catabolism, one in mitochondria and the other in peroxisomes [133].

A complex network of developmental and environmental cues controls the transcription, translation, and posttranslational processing of MCCase. The spatial and temporal accumulation patterns of *MCC-A* and *MCC-B* mRNAs are closely correlated during plant development [13]. In this developmental program, the expression of MCCase (measured as MCCase activity or accumulation of the subunits or their corresponding mRNAs) is at a maximum when catabolic processes are increased to maintain a carbon balance or export of carbon and energy. For example, MCCase expression is induced in senescing organs [13], in tissue-cultured cells [134], in plants undergoing autophagy [135], and during germination when seed storage proteins are being mobilized [13,132].

Transcription of the *Arabidopsis* MCCase subunit genes is induced when the carbon status of the plant is lowered, and this response appears to be mediated via a sugar-signaling pathway [135]. Furthermore, the sugar-signaling pathway that induces MCCase transcription requires biotin [136]. Additional characterization of the biotin requirement for MCCase expression indicates that biotin can control the translational or posttranslational regulation of MCCase expression [136]. For example, in tomato, the organ-specific differences in MCCase expression are determined by the posttranslational biotinylation of the MCC-A subunit [137]. Hence, whereas the MCC-A subunit accumulates to near equal levels in both roots and leaves, leaves express only 10% of the MCCase activity found in roots. This difference is due to the lower biotinylation status of the MCC-A subunit in the leaf.

Other biotin-containing proteins

Seed biotin-protein

Unusual biotin-containing proteins, SBPs, of about 50 to 65 kDa, accumulate specifically in the seeds or embryos of some plant species, including pea, soybean, carrot, and *Arabidopsis* [27,138–141]. These proteins are unusual for two major reasons. First, they accumulate late in embryogenesis and are quickly degraded upon seed germination [27,139,142,143]. Second, the amino acid sequence immediately surrounding the lysine residue that is biotinylated is not the consensus biotinylation site sequence found in other biotin-enzymes [138,139]. To date, no catalytic activity has been associated with any of these SBPs. Based on the finding that they accumulate in protein bodies [144], they are thought to store biotin in seeds.

Geranyl-CoA carboxylase

GCCase (EC 6.4.1.4) catalyzes the ATP-dependent carboxylation of the methyl group that is proximal to the carboxyl end of the monoterpene geranyl-CoA to form γ -carboxygeranyl-CoA. This enzyme was first detected and characterized from two genera of bacteria that are able to catabolize acyclic isoprenoids as a sole carbon source [128,129,145]. This carboxylation reaction enables the subsequent metabolism of γ -carboxygeranyl-CoA via a modified β -oxidation pathway, thereby allowing growth on acyclic monoterpenes [146,147]. The *Pseudomonas* enzyme has a molecular weight of about 550,000 and is composed of two distinct 75- and 63-kDa subunits. Only the larger is biotinylated.

The biotin-containing subunit of the maize GCCase is 122 kDa, and enzyme activity and this subunit are located in plastids. The enzyme has K_m of 64 μ M for geranyl-CoA, 0.6 mM for bicarbonate, and 8 μ M for ATP. The metabolic function of GCCase in plants is yet to be established. It may be required in the catabolism of acyclic isoprenoids, as in selected bacteria. Although initial reactions in the degradation of gibberellins, carotenoids, and phytol have been established in plants [148–152], little is known about the subsequent catabolism of these isoprenoids [148,153]. Characterization of the biochemical function(s) of GCCase in plants will likely reveal novel metabolism that is currently not well characterized. Ultimate confirmation of the presence of GCCase in plants awaits identification of the gene sequence.

Holocarboxylase synthetase

Holocarboxylase synthetase (HCS) catalyzes the biotinylation of biotin-containing proteins. Although HCS is not a biotin-containing protein, we include it in

this discussion, because all biotin-containing enzymes are dependent on it. HCS biotinylates the ϵ -NH₂ group of a unique lysine residue of biotin-containing proteins. With the exception of SBP, the amino acid sequence surrounding the lysine residue that is biotinylated is highly conserved among all biotin-containing proteins. Yet, it appears that HCS recognizes this lysine residue based on the context of the secondary structure within which the residue lies. In this context, it is not yet clear how SBP is biotinylated. The plant HCS has been partially characterized [154], and the activity of the enzyme has been reported in cytosol, mitochondria, and plastids [155], reflecting the multiple subcellular locations of biotinylated proteins. In *Arabidopsis* two genes that code for HCS have been characterized [156,157].

Conclusions

The discovery and characterization of biotin-containing enzymes has a history linked with the requirement of biotin in human and animal diets. Studies of these proteins began in animals in the 1950s, and by the 1970's, archetypal examples of most biotin-containing proteins had been biochemically characterized from animals and microbes. Relative to this rich history, characterization of biotin-containing proteins in plants is an endeavor with a much shorter history. Yet, within the past 10 years it has become apparent that studies of biotin-containing enzymes in plants offer insights into novel biochemical, metabolic, and evolutionary processes that are uniquely available for study in plants. For example, plants express biotin-containing enzymes that represent all three structural organizations of the functional domains. It appears that within the plant kingdom, one of these enzymes (the heteromeric ACCase) is being evolutionarily lost and replaced by another version of the same enzyme (homomeric ACCase). In addition, plants express a biotin-protein that has a unique BCC structural domain (SBP). Finally, studies of biotin-containing enzymes offer many biotechnological applications, particularly in relation to seed oil biosynthesis.

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