Evidence That Maize Acetyl-Coenzyme A Carboxylase Does Not Function Solely as a Homodimer†

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Acetyl-coenzyme A carboxylase (EC 6.4.1.2; ACCase) was purified from etiolated maize coleoptiles using cyclohexanedione affinity and DyeMatrex gel orange A, followed by anion-exchange chromatography. Purification yielded ACCase240, which was bound to orange A dye (purified 68×), and ACCase220, having no affinity for orange A dye (purified 79×). ACCase220 contained two biotinylated polypeptides with molecular mass of 220 and 85 kDa. The 85-kDa protein was separated from the 220-kDa protein and had ACCase activity. ACCase220 was composed of seven proteins as determined by native PAGE. Two-dimensional electrophoresis (native/SDS PAGE) of ACCase220 revealed that these seven native proteins share constituent subunits. These subunits of ACCase220 were cross-linked using dithiobis(succinimidylpropionate), and unassociated polypeptides were removed by YM100 ultrafiltration. Reduction of cross-linked ACCase resulted in the reappearance of multiple polypeptides originally observed by SDS PAGE. On the basis of these results, we provide the first evidence that gramineae ACCase does not function solely as a homodimer.

Keywords: *ACCase; quaternary structure; enzyme complex; chemical cross-linking; graminicide*

INTRODUCTION

Acetyl-coenzyme A carboxylase (EC 6.4.1.2; ACCase) is the first dedicated enzyme in the *de novo* fatty acid biosynthetic pathway and the target site for two commercial classes of grass-selective herbicides, the cyclohexanediones (CHDs) and (aryloxy)phenoxypropionates (AOPPs) (Secor and Cseke, 1988; Rendina et al., 1989; Burton et al., 1991). These two classes of herbicides have been shown to selectively inhibit ACCase of gramineae weeds and are therefore used for their control in broadleaf crops (Betts et al., 1992; Devine et al., 1992; Gronwald et al., 1992; Tardif et al., 1993). It has been hypothesized that inhibition of ACCase is not the sole mode of action of these herbicides (Shimabukuro and Hoffer, 1996); however, it has been demonstrated that there is a specific interaction of these herbicides with ACCase. Furthermore, AOPP- and CHD-resistant and susceptible grasses show differential levels of ACCase sensitivity to these herbicides (Walker et al., 1990; Tardif and Powles, 1994; Herbert et al., 1996).

ACCase catalyzes the ATP-dependent conversion of acetyl-coenzyme A to malonyl-coenzyme A via a dual active-site mechanism (Finlayson and Dennis, 1983). Acetyl-coenzyme A carboxylation is achieved by way of two distinct half-reactions. The first reaction involves the hydrolysis of ATP and carboxylation of the biotin cofactor. The second half-reaction involves the transfer of the carboxyl group from the biotin cofactor to acetyl-coenzyme A, resulting in the formation of malonyl-coenzyme A (Finlayson and Dennis, 1983). To facilitate this reaction, the bicarbonate-derived carboxyl group must be shuttled from the first active site to the second active site. The overall reaction can be separated into three distinct enzymatic functions: biotin carboxylation, carboxyl transfer, and acetyl-CoA carboxylation.

The ACCases of dicotyledonous plants exist as a multimeric enzyme complex (Kannangara and Stumpf, 1972; Wurtele and Nikolau, 1992; Alban et al., 1994; Konishi and Sasaki, 1994; Roesler et al., 1994, 1996; Konishi et al., 1996). Furthermore, Konishi et al. (1996) states that all monocotyledonous plants with the exception of the gramineae also have a multisubunit quaternary structure. The multimeric quaternary arrangement is similar to the prokaryotic system where the three enzymatic functions reside on separate, constituent proteins (Kannangara and Stumpf, 1972; Wurtele and Nikolau, 1992; Alban et al., 1994; Konishi and Sasaki, 1994; Roesler et al., 1994, 1996; Konishi et al., 1996). A homodimeric form also exists in many dicotyledonous and monocotyledonous plants. For example, Alban et al. (1994) reported that pea ACCase exists in two forms, the predominant form being the multimeric complex and the minor form being homodimeric. Furthermore, Dehaye et al. (1994) reported that the two forms of ACCase in pea have differential sensitivity to the AOPP and CHD herbicides, with the multimeric form being resistant and the homodimeric form being susceptible to these herbicides.

Currently, it is believed that gramineae species have only a homodimeric form of ACCase which is sensitive to AOPPs and CHDs, thereby imparting selectivity between gramineae and dicotyledonous species (Egli et al., 1993; Herbert et al., 1996; Konishi et al., 1996). In several grass species, it has been reported that isozymes of homodimeric ACCase exist and that these isozymes exhibit differential sensitivity to the AOPP and CHD herbicides (Egli et al., 1993; Konishi et al., 1996). However, Nikolau and Hawke (1984) reported that maize ACCase was composed of six 60−61-kDa constituent biotinylated proteins. Similarly, Evenson et al. (1994) reported that *Lolium multiflorum* ACCase might have an associated protein or another subunit that does not contain biotin. Furthermore, gramineae ACCase has not yet been purified to homogeneity, and consequently, no convincing evidence for the presence of a

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solely homodimeric quaternary arrangement has been presented. Low molecular mass (<100-kDa) proteins observed by SDS PAGE have been dismissed as either proteolytic degradation products or as ribulose-1,5-biphosphate carboxylase (RuBisCo) (Egin-Buhler et al., 1980; Nikolau and Hawke, 1984; Egli et al., 1993). The possibility exists that these contaminating proteins are dissociated constituent polypeptides of a large enzyme complex or their association is required for ACCase activity in gramineae species. It is also possible that these proteins represent other biotinylated carboxylases (or their constituent subunits) which can utilize acetyl-coenzyme A as a substrate.

In the present paper, we provide evidence for our hypothesis that maize ACCase does not function solely as a homodimeric enzyme as previously reported. We show that constituent polypeptides of an active ACCase can be cross-linked using a homobifunctional reducible reagent, stabilizing a large native enzyme complex. The DSP cross-linking reagent will covalently attach primary amine groups of proteins specifically associated within the 12-Å range of the DSP molecule (Lomant et al., 1976). Following cross-linking, contaminating proteins can be easily removed by methods such as ultrafiltration. Once contaminants have been removed, cross-linked proteins can be liberated and identified by cleaving the disulfide bridge of the cross-linking reagent and analyzing the proteins by SDS PAGE. Upon reduction of the cross-linked ACCase complex, multiple low molecular mass polypeptides associated within 12 Å were observed. In addition, we demonstrate that low molecular mass polypeptides are retained by an ultrafiltration membrane due to their association with a large protein complex. On the basis of these results, we believe maize ACCase has both a multimeric and homodimeric component.

MATERIALS AND METHODS

Enzyme Isolation. All reagents were purchased from Sigma unless otherwise noted. Spectra/Por7 dialysis membrane was obtained from Spectrum Medical Laboratory Products. ACCase was isolated from 7-day-old etiolated maize coleoptiles (Pioneer No. 3902). Coleoptiles were harvested (135 g) and homogenized at 4 °C in 200 mL of buffer A (50 mM Tricine−KOH, 1.0 mM EDTA, 10% (v/v) glycerol, 5 mM DTT, 15 mM $NaHCO_3$, pH 8.3). Homogenization was performed in a Waring blender. Homogenate was filtered through four layers of cheesecloth and one layer of miracloth (Calbiochem). Crude filtrate was centrifuged at 10 000*g* for 30 min at 4 °C. After centrifugation, the supernatant was removed and crystalline ammonium sulfate was added (over a 20-min period) to 30% saturation with gentle stirring at 4 °C. Following a 1-h incubation period, the solution was centrifuged as described above, the pellet was discarded, and crystalline ammonium sulfate was added (as described previously), bringing the supernatant to 50% saturation. Proteins were pelleted as described earlier, and the pellet was resuspended in a minimal volume of buffer A. The resuspended pellet was dialyzed (Spectra/Por7 50 000 MWCO) overnight against 3 × 1-L buffer A to remove ammonium sulfate prior to dye ligand affinity chromatography.

Affinity Purification. The solution containing the resuspended 50% ammonium sulfate pellet was applied to a 2.6 × 25-cm reactive blue 4 agarose dye affinity column (Sigma) which was previously equilibrated with buffer A. The column was washed with equilibration buffer (buffer A) until all unbound protein was washed through, as indicated by absorbance at 280 nm. Bound protein was eluted using a 0−1 M linear KCl gradient over 400 mL at a constant flow rate of 1.0 mL/min. Eluted protein was detected by absorbance at 280 nm using a Bio-Rad Econo system flow-through UV detector. Eluted fractions were collected and assayed for ACCase activity as described in the following section. Active ACCase fractions were pooled, concentrated, and desalted using an Amicon positive-pressure ultrafiltration apparatus equipped with a YM100 (100 000 MWCO) membrane. Desalted, concentrated, protein was applied to a cyclohexanedione affinity column. The inhibitor affinity column was made by covalently linking the cyclohexanedione (CHD) herbicide 2-[1-[((3-(carboxyphenyl)-2-propenyl]oxy)imino]propyl]-5-phenyl-3-hydroxy-2-cyclohexen-1-one to ω-aminohexyl Sepharose using 1-ethyl-3-[(dimethylamino)propyl]carbodiimide according to the manufacturer's instructions. A 10-mL ligand solution was added to 10 mL of 50% gel slurry. The optimal ligand concentration was 4 μmol/mL gel slurry. Following washing to remove unreacted ligand, the concentration of bound ligand was calculated by measuring the absorbance at 280 nm (ϵ_{280nm} = 250 000 M^{-1} cm^{-1}) of the supernatant following the coupling reaction. Inhibitor affinity purification was achieved by applying the desalted, concentrated ACCase active fraction onto a 1.5 × 5-cm CHD affinity column at a constant flow rate of 0.5 mL/min in buffer A. Unbound protein was washed off with 4 column volumes of buffer A as indicated by the baseline absorbance at 280 nm. Bound protein was eluted with 1 column volume of buffer B (buffer A + 1.0 M KCl). Eluted protein was collected and assayed for ACCase activity. Active ACCase fractions were pooled, concentrated, and desalted by positive pressure ultrafiltration (YM100). Concentrated, desalted CHD affinity column eluant was applied to a 2.6 × 20-cm DyeMatrex gel orange A column (Amicon) equilibrated with buffer A. The column was washed with 5 column volumes of buffer A to remove unbound protein. Unbound protein was collected and assayed for ACCase activity. ACCase active protein was pooled and concentrated by YM100 positive-pressure ultrafiltration. Bound protein was eluted with a 400-mL linear 0−1 M KCl gradient. Fractions were assayed for ACCase activity, and active fractions were pooled, concentrated, and desalted by positive-pressure ultrafiltration (YM100). Protein fractions were applied to a MacroPrep Q Econo-pac anion-exchange column (Bio-Rad). Protein was loaded onto the column, which was previously equilibrated in buffer A at a constant flow rate of 2.0 mL/min. After a 5-column-volume wash, the bound proteins were eluted with a 100-mL 0−1 M linear KCl gradient. Protein fractions were assayed for ACCase activity, and the active fractions were pooled, concentrated, and desalted as described before. The final ACCase preparations were stored frozen at −20 °C until required.

Purification in the Presence of Protease Inhibitors. To determine if low molecular mass species were proteolytic degradation products, the purification was performed in the presence of a protease inhibitor cocktail. The protease inhibitor cocktail consisted of pepstatin A (50 μg/mL), soybean trypsin inhibitor (50 μg/mL), phenylmethanesulfonyl fluoride (1 mM), phosphoramidin (20 μg/mL), bestatin (20 μg/mL), leupeptin (0.1 μg/mL), and chymostatin (1.0 μg/mL). ACCase purified in the presence of protease inhibitors was compared to ACCase purified in the absence of protease inhibitors on the basis of ACCase activity and the pattern of protein bands observed by native and SDS poly(acrylamide) gel electrophoresis.

Activity Assays. ACCase activity was measured by monitoring the production of heat and acid-stable [^{14}C]malonyl-coenzyme A from $NaH^{14}CO_3$ according to previously described methods (Burton et al., 1989, 1991; Alban et al., 1994; Evenson et al., 1994). Fixed ^{14}C was identified as malonyl-CoA by comigration of radioactive species with a nonlabeled malonyl-CoA standard on reversed-phase $KC_{18}F$ thin-layer chromatography plates (Whatman) using a 50% (v/v) CH_3CN mobile phase. Samples (100 μL) were mixed with 100 μL of reaction solution (50 mM Tricine, 5.0 mM $MgCl_2$, 20 mM DTT, 2.0 mM ATP, 14.97 mM $NaHCO_3$, 0.03 mM $NaH^{14}CO_3$ [3.7 × 10^7 Bq ^{14}C], pH 8.3) and preincubated for 10 min at 30 °C. Reactions were initiated by addition of 50 μL of 1.5 mM acetyl-coenzyme A to give a final concentration of 0.3 mM. Following a 10-min incubation, the reaction was quenched with 100 μL of 6 M HCl. Reaction tubes were evaporated to dryness at 80 °C and reconstituted with 100 μL of distilled deionized water.

Table 1. Summary of ACCase Purification from Etiolated Maize Coleoptiles

sample	vol, mL	[protein], mg/mL	amt of protein, mg	activity CPM[a]/mg protein	purification, -fold
homogenate	284	2.48	704	1 600	1
30−50% $(NH_4)_2SO_4$ cut	35	14	490	3 295	2.1
blue agarose affinity column (eluant)	20	1.74	35	22 207	14
CHD[b] affinity column (eluant); YM100 (retentate)	20	0.6	12	57 133	36
orange affinity column (eluant)[c]	4	0.0143	0.0572	109 090	68
orange affinity column (unbound)	8	1.9	15.2	28 084	18
MacroPrep Q column of orange affinity column unbound fraction[d]	7	0.35	2.5	126 314	79

[a] Counts per minute. [b] Cyclohexanedione. [c] Designated ACCase240. [d] Designated ACCase220.

Reconstituted samples were added to 5.0 mL of EcoLite scintillation cocktail, and the concentration of acid- and heat-stable ^{14}C was determined by liquid scintillation counting. Radioactive counts were directly proportional to the concentration of malonyl-coenzyme A.

Gel Electrophoresis. Both native and denaturing gel electrophoresis were performed for the analysis of ACCase purity and quaternary structure determination. Denaturing gel electrophoresis was performed in a Tris-glycine buffer system according to the methods of Laemmli (1970) using a discontinuous poly(acrylamide) slab gel (10% T, 2.6% C) with pH 6.8 stacking gel and pH 8.8 separating gel. Gels were run on both Mini Protean II and 20-cm Protean II systems. All gels were run at 170 V (constant voltage). Native gel electrophoresis was performed at a poly(acrylamide) concentration of 6% T and 2.6% C and a discontinuous system utilizing the same Tris buffer as described for the denaturing gel system. Gels were silver stained using the BioRad Silver Stain Plus kit according to the instructions of the manufacturer.

Two-Dimensional Gel Electrophoresis. Two-dimensional electrophoresis was performed using 6% native PAGE in the first dimension as described in the preceding section. Samples were dialyzed extensively to remove excess salt, and Triton X-100 was added to a final concentration of 0.02% (v/v). After electrophoresis in the first dimension, the lanes containing ACCase were excised. Representative lanes were silver stained, and the lanes to be subjected to second-dimension electrophoresis were incubated at 22 °C for 30 min in Laemmli sample buffer containing β-mercaptoethanol. Denatured, reduced, native PAGE lane containing ACCase was subjected to SDS PAGE (10%). The reduced gel slice was placed on top of a 16-cm discontinuous SDS PAGE slab gel and run as described in the previous section. Proteins were detected by silver staining as previously described. Biotinylated polypeptides were identified by avidin−horseradish peroxidase (avidin−HRP) blotting.

Detection of Biotinylated Polypeptides. For detection of biotinylated polypeptides, proteins were transferred to 0.02-μm poly(vinylidene difluoride) (PVDF, Bio-Rad) membranes and subsequently detected by probing with avidin−HRP (Pierce). Transfer was performed in cooled transfer buffer (15.6 mM Tris, 120 mM glycine, 10% (v/v) CH_3OH, pH 8.3) using the Protean II minigel apparatus (Bio-Rad) equipped with an electrotransfer apparatus. In order to detect biotinylated polypeptides of native ACCase, it was necessary to denature the enzyme after transfer with SDS. Proteins were electroblotted to PVDF membranes at 100 V (constant voltage) for 1 h at 4 °C. PVDF membranes were blocked using 3% (w/v) bovine serum albumin in phosphate buffered saline (10 mM phosphate, 15 mM NaCl, 10% (v/v) glycerol, pH 7.5) (PBS) for 30 min with shaking. Avidin−HRP was diluted 1:1000 in PBS and incubated with the membrane for 2 h. Membranes were washed 3 times with PBS, containing 0.05% (v/v) Tween 20. Biotinylated bands were visualized using the substrate 4-chloro-1-naphthol (30 μg/mL, Pierce). Substrate was prepared by dissolving premeasured tablets in 10 mL of methanol, which was added to 90 mL PBS. Immediately prior to development of the blot, 19.6 μL of 50% (v/v) H_2O_2 was added to the 4-chloro-1-naphthol solution. The reaction was quenched by washing the membrane in distilled deionized water. The results were recorded by photographing the dried PVDF membranes.

Chemical Cross-Linking of the Associated Proteins. Dithiobis(succinimidylpropionate) (DSP, Pierce) was used to cross-link proteins associated within 12 Å. Prior to cross-linking, the buffer was changed from tricine to phosphate by dialyzing (Spectra/Por7 50 000 MWCO) against 3 × 1-L PBS (10 mM phosphate, 15 mM saline, 10% (v/v) glycerol, pH 7.5) at 4 °C. Glycerol (10% (v/v)) was added following dialysis. Cross-linking was performed according to the manufacturer's directions, using PBS and DSP solubilized in dry dimethyl sulfoxide (DMSO). Cross-linked proteins were subjected to SDS PAGE and native PAGE to determine the nature of the protein interactions. DSP cross-link bridges were cleaved using either dithiothreitol (DTT) or β-mercaptoethanol for 2 min at 100 °C prior to electrophoretic analysis. When the reaction volume was greater than 5 mL, the cross-linked protein solution was concentrated by YM100 positive-pressure ultrafiltration to remove unreacted cross-linking reagents and unassociated proteins.

RESULTS AND DISCUSSION

Following blue agarose and CHD affinity chromatography, ACCase activity was separated into two active preparations, hereafter designated as ACCase220 and ACCase240. ACCase240 was purified 68-fold (Table 1) by binding to an orange A column, eluting with KCl, followed by concentration and desalting by ultrafiltration (YM100). ACCase220 did not bind to the orange A column. It was further purified by MacroPrep Q anion-exchange chromatography. Subsequent concentration and desalting using YM100 ultrafiltration of active protein eluted from the anion column yielded ACCase220, which was purified 79-fold (Table 1).

ACCase240 contained biotinylated polypeptides with estimated molecular mass of approximately 240 and 85 kDa (Figure 1, lane 5). ACCase220 contained constituent biotinylated proteins with estimated molecular mass of approximately 220 and 85 kDa (Figure 2). Molecular masses were estimated by extrapolation from a plot of relative mobility vs log(MW) and were consistent with the molecular masses previously reported for maize ACCase (Egli et al., 1993). In addition to biotinylated proteins, both ACCase220 (Figure 3, lane 6) and ACCase240 (Figure 3, lane 4) contained several nonbiotinylated low molecular mass proteins (see Figure 4 for elution profiles of ACCase220 and 240, respectively). These low molecular mass proteins were not degradation products since the results in the presence of proteolytic enzyme inhibitors were the same as those shown in Figure 3. Other researchers, using different monocotyledonous and dicotyledonous species, also found low molecular mass polypeptides that were not the result of proteolytic degradation (Pyke et al., 1991; Wurtele and Nikolau, 1992; Egli et al., 1993; Gornicki et al., 1994). For example, Pyke et al. (1991) extracted ACCase from pea seeds directly into Laemmli denaturing buffer, and found low molecular mass proteins.

Prior to separation of ACCase220 and ACCase240, all ACCase activity and multiple low molecular mass proteins in the homogenate were specifically bound to

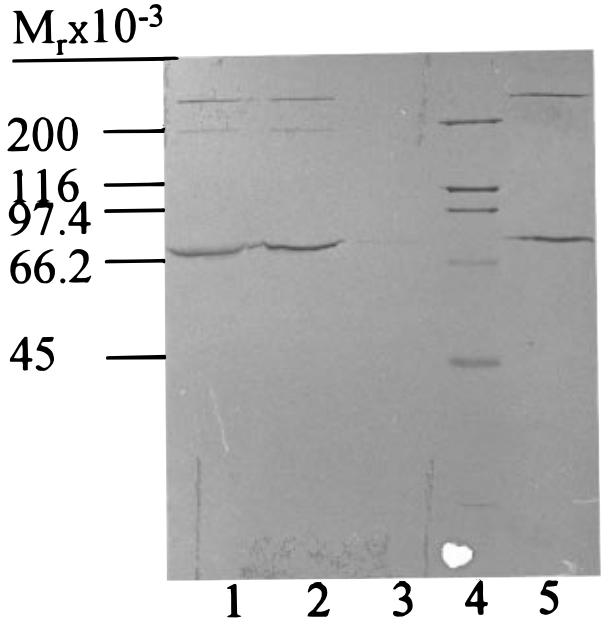

Figure 1. Western blot of ACCase showing three biotinylated polypeptides corresponding to apparent molecular mass of 85, 220, and 240 kDa. Lane 1, blue column eluant; 2, CHD column eluant; 3, YM100 filtrate of CHD column eluant; 4, biotinylated molecular mass markers; 5, orange column eluant (ACCase240). Biotinylated polypeptides were detected with avidin−HRP using 4-chloro-1-naphthol chromogenic substrate.

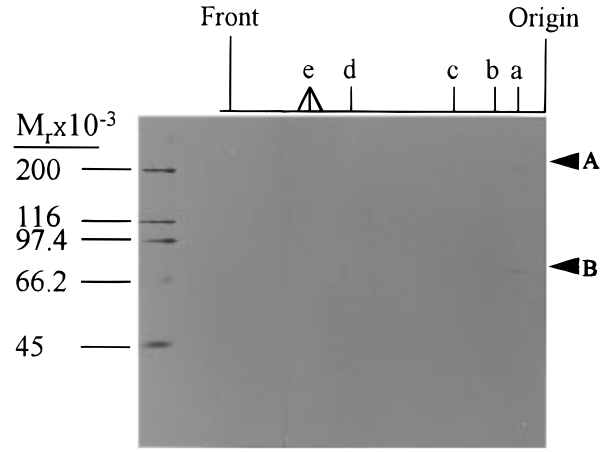

Figure 2. Avidin−HRP blot of two-dimensional electrophoresis of ACCase220. First native dimension origin and dye front are shown at the top of the figure. Major native PAGE bands are labeled a, b, c, d, and e at the top of the figure in order of increasing R_f value. Second dimension, 10% SDS PAGE was performed under reducing conditions, migration distance of molecular mass markers are indicated. Biotinylated proteins were detected with avidin−HRP conjugate and 4-chloro-1-naphthol substrate (A, 220 kDa; B, 85 kDa).

the CHD affinity column. The binding of the low molecular mass proteins was determined to be specific because these proteins were not removed by repeated washing of the affinity columns with three different solvent systems (dioxane, KCl, CH_3CN) as indicated by baseline absorbance at 280 nm. Therefore, low molecular mass polypeptides may be dissociated proteins of a larger complex which when associated has affinity for the CHD column. When eluted, the complex dissociates, giving rise to the identical low molecular mass proteins observed in the wash fractions. Gornicki and Haselkorn (1993) reported similar results in monocotyledonous wheat plants. They observed low molecular mass biotinylated polypeptides, which they assumed to be degradation products. Furthermore, they stated these

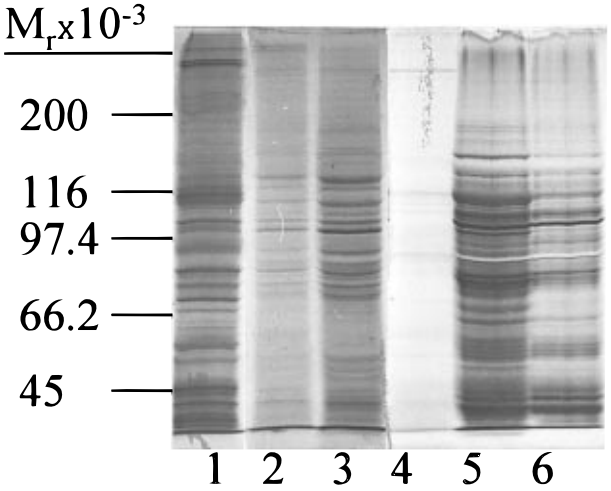

Figure 3. SDS−PAGE (10%) of ACCase purification fractions. Lanes: 1, crude homogenate; 2, blue column eluant; 3, CHD column eluant; 4, orange column eluant (ACCase240); 5, orange column unbound fraction; 6, MacroPrep Q column active fraction (ACCase220). All lanes are silver stained, and fractions were desalted and concentrated by YM100 positive-pressure ultrafiltration. Positions of molecular mass markers are indicated adjacent to lane 1.

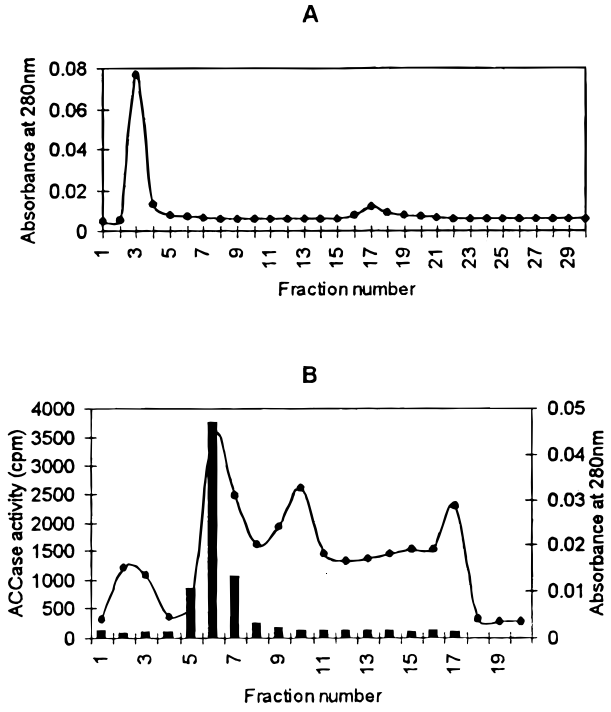

Figure 4. Elution profiles for the purification of ACCase220 and ACCase240 by DyeMatrex gel orange A and MacroPrep Q anion-exchange chromatography. Panel A, plot of absorbance at 280 nm vs fraction number for the elution of ACCase240 from the DyeMatrex gel orange A dye ligand affinity column. Panel B, plot of absorbance at 280 nm (—) vs fraction number for the purification of ACCase220 by MacroPrep Q anion-exchange chromatography. ACCase activity (|) is also shown for each fraction.

degradation products might have retained their ability to form the ca. 500-kDa complex. We disagree with the interpretation of Gornicki and Haselkorn (1993) and propose that the low molecular mass proteins found in their experiments and ours were actually subunits of a native ACCase complex.

To confirm our hypothesis that low molecular mass proteins were actually subunits of a native ACCase complex, successive ultrafiltration through a 100-kDa

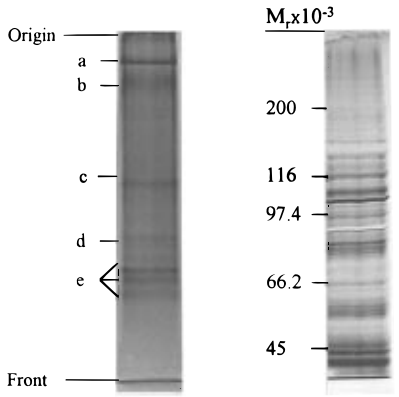

Figure 5. Native PAGE (6%) of eluant from the MacroPrep Q anion-exchange column (ACCase220) and 10% SDS PAGE of ACCase220. Both lanes were silver stained. Major bands present in the native PAGE are labeled a, b, c, d, and e in order of increasing R_f. All samples were concentrated and desalted by YM100 ultrafiltration prior to gel electrophoresis. Positions of molecular mass markers are indicated adjacent to the SDS PAGE lane.

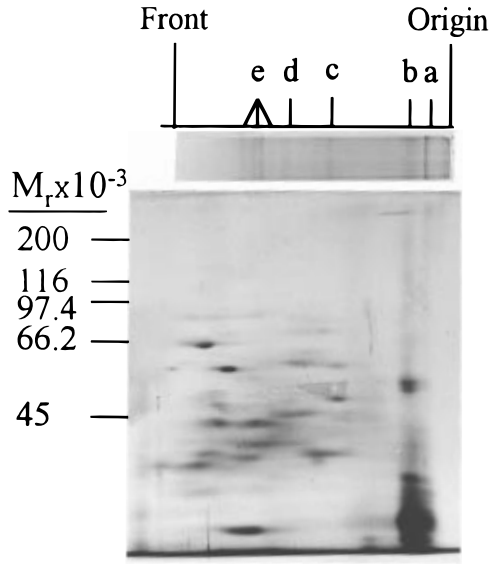

Figure 6. Two-dimensional (native/SDS PAGE, respectively) electrophoresis of ACCase220. Silver-stained first native dimension lane, origin and dye front are shown at the top of the figure. Approximate positions of proteins in the native dimension are indicated at the top of the figure and denoted as before in Figure 2. Second dimension, 10% SDS PAGE was performed under reducing conditions, and migration distance of molecular mass markers are indicated.

MWCO membrane was conducted. Figure 3, lane 6 shows low molecular mass proteins present in the ACCase220 preparation which were subjected to four YM100 ultrafiltration washes (one after each chromatography step). The same proteins were found in both the YM100 filtrate and retentate fractions. Only when harsh reducing and denaturing (DTT and SDS) conditions were applied could substantial amounts of low molecular mass proteins be forced through the YM100 membrane. After repeated washing of the ACCase within the YM100 ultrafiltration cell, the concentrated filtrate contained a single biotinylated protein with molecular mass of 85 kDa (Figure 1, lane 3). This 85-kDa biotinylated protein had ACCase activity (specific activity 35 294 CPM/mg of protein). Although it may be argued that this 85-kDa biotinylated protein may be a constituent protein of 3-methylcrotonyl coenzyme A carboxylase (MCCase) as reported by Dehaye et al. (1994), this polypeptide also has ACCase activity. Nikolau and Hawke (1984) reported that a maize leaf extract had ACCase activity in the absence of high molecular mass biotinylated proteins. They reported that maize ACCase consisted of six 60−61-kDa biotinylated proteins in a α_6 quaternary arrangement. Ashton et al. (1994) also found ACCase activity in maize in the absence of high molecular mass biotinylated proteins and a biotinylated protein with molecular mass of ca. 80 kDa. Burns et al. (1995) found a multimeric ACCase from *Helicobacter pylori* that maintained its association through purification using YM100 ultrafiltration. On the basis of the retention of the low molecular mass biotinylated protein (24 kDa), Burns et al. (1995) concluded that ACCase was a multimeric complex. Our results and those of Nikolau and Hawke (1984), Ashton et al. (1994), and Burns et al. (1995) support our hypothesis that maize ACCase does not function solely as a homodimer.

Native PAGE showed a pattern of seven protein bands after elution from the MacroPrep Q anion-exchange column (ACCase220; Figure 5). Native PAGE bands had R_f values (labeled a−e) of 0.09, 0.13, 0.43, 0.59, 0.69, 0.72, and 0.76, respectively. SDS PAGE analysis of the same preparation (ACCase220 eluted from MacroPrep Q anion-exchange column; Figure 5) shows that the native bands are composed of more than seven polypeptides; i.e., one or more of the native bands are composed of the previously mentioned multiple low molecular mass proteins. This is shown in more detail by two-dimensional electrophoretic analysis of ACCase220 (Figure 6). The band labeled a (Figure 6) gave rise to many low molecular mass bands after SDS PAGE in the second dimension. Furthermore, the avidin−HRP blot (Figure 2) of the ACCase220 two-dimensional gel shows that of the ca. 15 polypeptides (Figure 6, lane a), 2 are biotinylated and have molecular masses of approximately 220 and 85 kDa, respectively (Figure 2). It is important to remember that these low molecular mass polypeptides should have been removed by the YM100 ultrafiltration membrane but they were not. Furthermore, when native proteins b through e are run in the second dimension (SDS PAGE), some polypeptides have R_f values the same as polypeptides derived from band a (Figure 6). Therefore, the native bands in the second dimension of Figure 6 may be derived from common constituent subunits. Furthermore, we believe the seven native bands (Figures 5 and 6) represent different degrees of dissociation of the ACCase complex; when run in the second dimension (SDS PAGE), each of the seven native bands dissociates into the constituent polypeptides.

Based on the previous results, DSP cross-linking experiments were done to determine which proteins were associated with the ACCase220 complex. Cross-linking maize ACCase resulted in a large protein complex that was visualized by SDS PAGE under nonreducing conditions (Figure 7, lane 2). Reduction of the cross-linked complex following removal of unassociated proteins by YM100 ultrafiltration resulted in multiple protein bands associated within 12 Å (Figure 7, lane 1). These results indicated that the low molecular mass proteins were specifically associated to form a multimeric complex with a molecular mass greater than 100 kDa as determined by YM100 ultrafiltration.

It has been reported that dicotyledonous and all monocotyledonous plant species except gramineae contain ACCase in the multisubunit form (Kannangara and

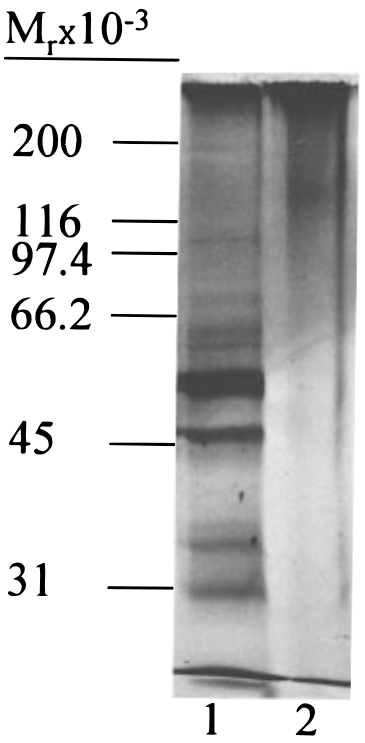

Figure 7. ACCase active CHD affinity column eluant cross-linked with DSP subjected to reducing conditions and cross-linked polypeptides compared to those in noncross-linked ACCase control by 10% SDS PAGE. Lanes: 1, reduced cross-linked ACCase; 2, nonreduced cross-linked ACCase.

Stumpf, 1972; Alban et al., 1994; Chapman-Smith et al., 1994; Dehaye et al., 1994; Shorrosh et al., 1995; Konishi et al., 1996). Furthermore, on the basis of our results, we hypothesize that ACCase isolated from maize coleoptiles does not function solely as a homodimer. Low molecular mass polypeptides dismissed as contaminants by other researchers may be polypeptides associated with the ACCase complex. Two forms of ACCase, ACCase220 and ACCase240, can be separated by Dye-Matrex gel orange A affinity chromatography. ACCase220 shows electrophoretic characteristics typical of a multimeric enzyme complex. Furthermore, ultrafiltration experiments show that low molecular mass polypeptides are associated to form a native complex with MW greater than 100 kDa. Cross-linking studies performed on ACCase show that there are multiple proteins associated within 12 Å. Finally, a YM100 filtrate was shown to have an ACCase activity independent of the 220- or 240-kDa biotinylated polypeptides, which were previously believed to be essential for ACCase activity. These results provide evidence that supports the hypothesis that maize acetyl-coenzyme A carboxylase does not function solely as a homodimer.

The purification and characterization of ACCase is similar to the case of transcarboxylase. Transcarboxylase has a complicated quaternary structure that undergoes varying degrees of dissociation and reassociation depending on buffer composition (Wood et al., 1975; Wood and Zwolinski, 1976; Wood and Barden, 1977). After extensive sedimentation and ultracentrifugation experiments, it was determined that transcarboxylase was composed of a large central subunit surrounded by three peripheral subunits. The 145-kDa peripheral subunits were composed of two subunits, and one of those subunits contained two 12-kDa biotinylated polypeptides (Wood et al., 1975). Therefore, this large multisubunit enzyme which upon initial purification attempts appeared impure was actually a purified enzyme in various states of dissociation. Furthermore, human ACCase has been found to be a protofilament composed of heterodimeric subunits, and multimeric forms have been found in prokaryotes, dicotyledonous plant species and all monocotyledonous plant species, except grasses. Based on our results, those of others working on monocotyledonous plants (Gornicki and Haselkorn, 1993; Ashton et al., 1994; Evenson et al., 1994; Nikolau and Hawke, 1994), and the fact that a homodimeric form has never been purified and isolated in grasses, it seems unlikely that a multimeric form of ACCase does not exist in grasses.

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