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PURIFICATION AND CHARACTERIZATION OF ACETYL-CoA CARBOXYLASE FROM DEVELOPING SOYBEAN SEEDS*

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Abstract—Acetyl-CoA carboxylase from two lines of soybean (*Glycine max*) seeds has been purified to apparent homogeneity. The procedure included affinity chromatography of the enzyme on avidin-monomer-Sepharose 4B. The enzyme from both lines showed a single band on polyacrylamide gel electrophoresis. On sodium dodecyl sulphate-polyacrylamide gel electrophoresis, the enzyme from experimental line 9686 showed a single protein band having the M_r 240 000. The enzyme from the commercial line Wayne, however, showed three protein bands having the M_rs 240 000, 65 000 and 58 000, respectively. High concentrations of the enzyme were required for stability as well as the presence of dithiothreitol, glycerol and Triton X-100. The enzyme was active over a wide pH range, with an optimum at 8.2 for 9686 and 7.5 for Wayne. The enzyme from both 9686 and Wayne showed absolute specificity for acetyl-CoA as a substrate and this could not be replaced by propionyl-CoA, butyryl-CoA, hexanoyl-CoA or 3-methylcrotonyl-CoA. At the optimum pH the apparent K_m values for the substrates were: bicarbonate, 1.13 mM; acetyl-CoA, 0.32 mM; ATP, 0.46 mM for the Wayne carboxylase and bicarbonate, 1.56 mM; acetyl-CoA, 0.17 mM; ATP, 0.14 mM for the 9686 enzyme. Citrate, at higher concentrations, was strongly inhibitory. Both ADP and AMP inhibited the enzyme from 9686 and Wayne. The enzyme from both 9686 and Wayne did not appear to be highly regulated by cellular metabolites.

INTRODUCTION

The carboxylation of acetyl-CoA to form malonyl-CoA is an ATP-dependent reaction catalysed by acetyl-coenzyme A carboxylase (acetyl-coenzyme A: bicarbonate ligase [ATP], EC 6.4.1.2). This enzyme offers a potential site for metabolic control as it catalyses the first step at which carbon precursors are committed to the biosynthesis of fatty acids and flavonoids. The regulation of acetyl-CoA carboxylase activity would govern the entry of precursors into the biosynthetic pathways, and the subsequent demands on energy reserves for the biosynthetic process. Extensive studies over the last 20 years have led to an understanding of the role of this enzyme in the regulation of *de novo* fatty acid biosynthesis in *Escherichia coli*, yeast and various mammalian sources [1–3]. The structure of acetyl-CoA carboxylase from plant tissues is still unclear. The enzyme has been isolated from photosynthetic tissues [3–7] and non-photosynthetic tissues [8–13]. All the preparations of acetyl-CoA carboxylase have been found to differ in their subunit structures depending on the preparation and techniques employed. The enzyme from photosynthetic tissue [4] is composed of three reversibly dissociable protein components similar to those of the *E. coli* carboxylase [14]. In contrast, the carboxylase from non-photosynthetic tissues appears to be soluble and has been purified as an active complex from both wheat germ [9, 11], barley embryos [8] and castor oil seeds [15]. However, the enzyme purified from avocado mesocarp plastids [5], while easily solubilized, may be membrane bound, so that acetyl-CoA carboxylase from non-photosynthetic tissues may be associated with the membranes of plastids in a similar manner to the chloroplast enzyme. In a previous paper [16] we reported the characterization of acetyl-CoA carboxylase from two lines of soybean seeds; the commercial genotype Wayne and the experimental genotype 9686. This report describes the purification and kinetic properties of acetyl-CoA carboxylase from the developing seeds of two lines of soybean.

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RESULTS AND DISCUSSION

Reaction characteristics

The incorporation of $H^{14}CO_3$ into ^{14}C-labelled malonyl-CoA by acetyl-CoA carboxylase required Mg^{2+}, acetyl-CoA, $H^{14}CO_3$ and ATP, and was inhibited by avidin. Under optimum conditions the enzyme assay was linear up to 50 μg protein in both Wayne and 9686, and for at least 15 min. Analysis of the reaction products by thin layer chromatography [17] showed malonyl-CoA as the only ^{14}C product formed. Presence of 0.2% Triton X-100 in the homogenizing media, was essential to obtain maximal activity. Manganese could not replace Mg^{2+} in both Wayne and 9686 as an essential activator of the enzyme.

Enzyme purification

The enzyme has been purified employing ammonium sulphate precipitation, and affinity chromatography on avidin-monomer-Sepharose 4B. In the final chromatographic step, all of the carboxylase activity was retained by avidin-monomer-Sepharose 4B, in contrast to the bulk of the protein. Acetyl-CoA carboxylase was eluted as a sharp peak after application of buffer D. This affinity chromatography step resulted in a 302-fold purification of the 9686 carboxylase and 123-fold purification of the Wayne carboxylase from the $(NH_4)_2SO_4$ step (Tables 1 and 2). Employing avidin-monomer-Sepharose 4B, Egin-Buhler and Ebel [10] achieved a 1000-fold overall purification of acetyl-CoA carboxylase from irradiated parsley cells.

Biotin content

The covalent attachment of the biotin prosthetic group to the carboxylase is indicated by the binding of the enzyme to avidin-Sepharose 4B and by the complete inhibition of enzyme activity after preincubation with 3 μM avidin.

Kinetic constants

The apparent K_m values for Wayne and 9686 carboxylase were obtained using enzyme purified by affinity chromatography. Plots of the carboxylation rates versus the concentrations of all substrates tested (acetyl-CoA, bicarbonate, ATP) resulted in hyperbolic curves. The following K_m values were calculated from linear double-reciprocal plots: acetyl-CoA, 0.32 mM; bicarbonate, 1.13 mM; ATP, 0.46 mM for Wayne acetyl-CoA carboxylase and acetyl-CoA, 0.17 mM; bicarbonate, 1.56 mM; ATP, 0.14 mM for 9686 acetyl-CoA carboxylase. The carboxylase from the two genotypes had different K_m values for the substrates, thus exhibiting different affinities. However, the carboxylase from different tissues does exhibit different K_m values. The enzyme from developing castor seeds [15] had K_{ATP}, 0.1 mM; $K_{HCO_3^-}$, 3.0 mM and $K_{acetyl\text{-}CoA}$, 0.05 mM. The parsley carboxylase [10] had K_{ATP}, 0.07 mM; $K_{HCO_3^-}$, 1.0 mM and $K_{acetyl\text{-}CoA}$, 0.15 mM. GTP, TTP, CTP, UTP and ITP at 0.2 mM were tested as substrates in place of ATP, but no $H^{14}CO_3^-$ incorporation was observed with the carboxylase from Wayne or 9686. In the case of castor oil seed enzyme [15] $H^{14}CO_3^-$ incorporation was observed in the presence of UTP, at a rate of 2.5% of that found with ATP.

Substrate specificity

The substrate specificity of the Wayne and 9686 carboxylase was tested using the straight-chain acyl-CoA esters, acetyl-CoA, propionyl-CoA, butyryl-CoA and hexanoyl-CoA, and the branched chain ester, 3-methylcrotonyl-CoA as substrates. At concentrations of 0.3 mM no carboxylation was observed. In parsley, propionyl-CoA and butyryl-CoA showed 60% and 15% carboxylation, respectively [10].

Effect of pH and temperature

The effect of pH on the carboxylation rate was tested between pH 6.5 and pH 9.2 using Na phosphate, Tris–HCl and glycine–NaOH buffers. As reported earlier [16], the carboxylase from Wayne had a pH optimum of 8.2 while the carboxylase from 9686 had a pH optimum of 7.5.

Under standard assay conditions, the carboxylase activity was measured over the temperature range 25–40°. The activity was found to be markedly affected by temperature, the optimum being 35–40° for both Wayne and 9686 carboxylase. The standard assay was performed at 37°. The parsley enzyme had an optimum of 45° [10].

Effect of metabolites

The effect of various metabolites tested on acetyl-CoA carboxylase from Wayne and 9686 are recorded in Table 3. Of all the metabolites tested, glycine was the only

Table 1. Purification of 9686 acetyl-CoA carboxylase by affinity chromatography on avidin-monomer-Sepharose 4B

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude homogenate	630	20.0	0.03	—	100
Ammonium sulphate (10–40%) fraction	130	18.6	0.15	5	93
Affinity chromatography	0.84	7.6	9.08	302	38

Table 2. Purification of Wayne acetyl-CoA carboxylase by affinity chromatography on avidin-monomer-Sepharose 4B

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude homogenate	150.5	12.5	0.08	—	100
Ammonium sulphate (10–40%) fraction	29.3	11.5	0.39	5	92
Affinity chromatography	0.56	5.5	9.85	123	44

Table 3. Effect of metabolites on the activity of acetyl-CoA carboxylase

Metabolite	Relative rate	
	Wayne	9686
None	100	100
Glucose-6-phosphate	100	100
Fructose-6-phosphate	103	100
Dihydroxy acetone phosphate	100	100
3-phosphoglycerate	100	100
Pyruvate	102	110
Phosphoenolpyruvate	110	98
Fructose-1,6-diphosphate	112	111
Malate	102	100
Glycine	197	146
NADH	100	100
Citrate	48	50
Acetate	100	100
Pyrophosphate	100	100

one which activated the carboxylase both from Wayne and 9686. However, the rape seed carboxylase was not affected by glycine. Citrate at 10 mM inhibited both Wayne and 9686 carboxylase. This inhibition by citrate, an allosteric activator of the animal carboxylase [14], could be relieved by the addition of equimolar concentrations of Mg^{2+}. The same effect was also observed for wheat germ carboxylase [18] and castor oil seed carboxylase [15]; however, the activity of the carboxylase from either spinach or avocado was stimulated by 3 mM citrate [5]. There was no inhibition by either NADH or NADPH as has been reported for the castor oil seed enzyme [15].

Acetyl-CoA carboxylase from rat liver [19] and crude spinach chloroplast preparations [20] was activated by coenzyme A. Crude plastid preparations from castor oil seeds [15] when incubated with 0.1 mM CoA for 20 min at 4° resulted in a 1.5-fold stimulation of the enzyme rate. However, CoA had no effect on acetyl-CoA carboxylase either from Wayne or 9686.

Molecular weight and subunit composition

Analysis of purified acetyl-CoA carboxylase both from Wayne and 9686 by PAGE under non-denaturing conditions gave rise to only one protein band in each case and suggested that the carboxylase was homogeneous. Analysis of the purified enzyme by SDS-PAGE gave rise to a single protein band in case of 9686 and three protein bands in case of Wayne.

The M_r of the biotinyl subunit of purified acetyl-CoA carboxylase has been determined in barley embryo [8], parsley culture [10], wheat germ [9], maize leaves [6], and leaves of C_3 and C_4 plants [3]. Biotin-containing peptide of M_r 240000 was reported in wheat germ [3], barley embryos [8] and parsley culture [9]. Maize leaf purified acetyl-CoA carboxylase contained a biotinyl subunit of 60000 [6]. In leaves of *P. sativum*, *A. porrum*, sorghum and *Z. mays*, four biotinyl proteins were reported with M_rs of 62000, 51000, 34000 and 32000 [6] respectively. In soybean, the experimental genotype 9686 had a single protein band of M_r around 240000, while the commercial genotype Wayne had three protein bands corresponding to M_r 240000, 65000 and 58000 respectively. In all the leaves examined the M_r 240000 biotinyl protein was not detected [3]. Thus the soybean acetyl-CoA carboxylase resembles the wheat germ, barley embryo and parsley cell culture acetyl-CoA carboxylase. Possibly, leaf acetyl-CoA carboxylase has a biotinyl subunit of a different size. Moreover, acetyl-CoA carboxylase in leaves is required in a number of different cell types to supply malonyl-CoA for at least six different biosynthetic pathways, and because purified acetyl-CoA carboxylase activities from different tissues and species differ in their molecular organizations, there is a possibility that these activities represent isoenzymes of acetyl-CoA carboxylase.

Effect of ADP and AMP

Eastwell and Stumpf [21] reported that both ADP and AMP inhibited acetyl-CoA carboxylase and that they were competitive inhibitors with respect to ATP. They showed that the crude extracts of wheat germ and spinach chloroplasts had ATPase and adenylate kinase activity which resulted in rapid hydrolysis and interconversion of ATP to yield ADP and AMP. This had a dramatic effect on the accuracy of the assay for acetyl-CoA carboxylase activity in crude preparations. In our experiments with acetyl-CoA carboxylase from soybeans, the assay of acetyl-CoA carboxylase did not follow ideal zero-order kinetics when assayed in a crude extract. When the crude extract was diluted, the decrease of acetyl-CoA carboxylase activity during the pre-incubation period with ATP was substantially reduced. In contrast, the partially purified acetyl-CoA carboxylase was insensitive to preincubation with ATP, and the assay remained linear over a much greater range of enzyme concentrations. This was also reported for wheat germ [21]. The extent to which these interconversion of adenylate nucleotides affected acetyl-CoA carboxylase activity was revealed by determining the effects of ADP and AMP on the purified enzyme from both Wayne and 9686. Interestingly, the results from these experiments showed that both ADP and AMP were inhibitory. Distinct differences were evident in the kinetic constants of 9686 and Wayne acetyl-CoA carboxylase, but in both cases, the enzymes were under the control of relative adenylate nucleotide concentrations.

Conclusions

From the data presented it appears that the soybean carboxylase resembles the wheat germ, castor oil seed, parsley and barley embryo acetyl-CoA carboxylase. It is not clear, however, whether citrate activation is a property common to all plant acetyl-CoA carboxylases. Burton and Stumpf [18] obtained 89% inhibition of the wheat germ acetyl-CoA carboxylase in the presence of 30 mM citrate, this being relieved when the citrate concentration was decreased to 5 mM; isocitrate (5 mM) produced a 10% activation. Heinstein and Stumpf [1] on the other hand reported that the wheat germ enzyme was not affected by 4 mM isocitrate and that higher concentrations were inhibitory. The castor oil seed enzyme too was inhibited by 5 mM citrate which could be relieved by adding equimolar concentrations of Mg^{2+}. However, the activity of the carboxylase isolated from either spinach or avocado was stimulated by 3 mM citrate [5].

The plant acetyl-CoA carboxylases seems to differ in

their subunit composition. The wheat germ [9], barley embryo [8] and parsley cell culture [10] have a 240000 subunit as does the soybean carboxylase. However, the leaf acetyl-CoA carboxylase does not have the 240000 subunit. Possibly the leaf acetyl-CoA carboxylases have a biotinyl subunit of different size. The enzyme from the two lines of soybean too, differ in their subunit composition.

EXPERIMENTAL

Materials. $NaH^{14}CO_3$ was purchased from Amersham International; CNBr-activated Sepharose 4B, and small and large M_r protein standards from Pharmacia; acetyl-CoA, propionyl-CoA, butyryl-CoA, hexanoyl-CoA, 3-methylcrotonyl-CoA, avidin and biotin from Sigma. All other reagents were of analytical grade.

Two lines of soybean plants (*Glycine max* L. Merr.) varying in linolenic acid content from 4 to 10%, were grown in the greenhouse, and seeds collected from low linolenic acid experimental line (9686) and high linolenic acid commercial variety (Wayne) were used for the study.

Buffers. (A) 0.1 M Tris–HCl, pH 8.2; (B) 0.1 M Tris–HCl, pH 8.2 containing 0.5 M NaCl and 2 mM EDTA; (C) buffer B, containing 2.5 mM DTT; (D) 0.05 M Tris–HCl, pH 8.2 containing 5% glycerol, 0.5 M NaCl, 2.5 mM DTT and 0.6 mM biotin; (E) 0.1 M Tris–HCl, pH 8.2 containing 1 mM phenylmethanesulphonyl fluoride, 2 mM EDTA, 5 mM DTT and 0.2% Triton X-100; (F) 0.1 M glycine–HCl, pH 2.0; (G) 0.01 M KPi buffer, pH 7.

Acetyl-CoA carboxylase activity was determined by measuring the incorporation of $NaH^{14}CO_3$ into malonyl-CoA. The incubation mixture contained, in a total vol. of 150 μl, 44 mM Tris–HCl, pH 7.5 or 8.2, 2 mM Na_2ATP, 5 mM $MgCl_2$, 1 mM DTT (dithiothreitol), 10 mM $NaH^{14}CO_3$ (1 μCi/μmol), 0.5 mM acetyl-CoA and enzyme protein (30–40 μg). The mixture was preincubated for 4 min at 37°. The reaction was started by the addition of acetyl-CoA preincubated for 1 min at 37°. After 5 min at 37°, the reaction was terminated by adding 60 μl of 6 M HCl. A portion of the mixture (100 μl) was dried on glass fiber disks for 1 hr and the acid-stable radioactivity was counted using a scintillator (4 g PPO and 50 mg POPOP in 1 l. toluene) in a Beckman liquid scintillation counter. Blanks without acetyl-CoA was included in every assay.

Protein was determined by the method of ref. [22] after precipitation with trichloroacetic acid using bovine serum albumin as a standard.

Monomeric-avidin-Sepharose 4B was prepared by the method of ref. [23]. CNBr-activated Sepharose 4B was washed with 1 mM HCl (200 ml/g dry gel) and 50 ml of buffer G. The gel was then suspended in 40 ml of buffer G and added to 10 mg of avidin dissolved in 20 ml of buffer G. The suspension was left overnight in cold with gentle agitation. The gel was filtered and washed with 100 ml of 1 M ethanolamine-HCl, pH 7, followed by 290 ml of 0.01 M buffer G. The gel was poured into a small column and washed with 6 M guanidine-HCl in 0.2 M KCl–HCl, pH 1.5. The column was left overnight to ensure complete dissociation. The column was washed with an additional 5 ml of guanidine-HCl and then with 15 ml buffer G until the absorbance at 280 nm was less than 0.01. The column was then washed with 1 mM biotin and with 10 vols of buffer F to remove biotin on the loose sites. The column was then equilibrated with buffer C.

Enzyme purification. Seeds were removed from the pods, and enzyme extracts prepared by grinding in a chilled mortar with pestle at 4°. Seeds were homogenized in 100 mM Tris–HCl, pH 8.2, containing 1 mM phenylmethanesulphonyl fluoride, 2 mM EDTA, 5mM DTT and 0.2% Triton X-100. The homogenate was filtered through two layers of muslin cloth and centrifuged at 5000 *g* for 20 min. The supernatant formed the crude fraction. The supernatant was brought to 10% satn with $(NH_4)_2SO_4$ and allowed to stand in cold for 2 hr. The supernatant obtained after centrifugation at 15000 *g* for 30 min was brought to 40% satn with $(NH_4)_2SO_4$ and allowed to stand for another 2 hr. After 2 hr the suspension was centrifuged for 30 min at 15000 *g* and the pellet collected. The pellet was redissolved in minimal vol. of buffer C. This was clarified by centrifugation at 10000 *g* for 20 min.

The clear protein soln was applied to an avidin-monomer-Sepharose 4B column (1.5 × 6 cm) previously equilibrated with buffer B. After the column had been extensively washed with buffer C elution of acetyl-CoA carboxylase was achieved with 20 ml of buffer D. Fractions of 2 ml each were collected. Fractions highest in enzyme activity were pooled. The combined fractions were passed through disposable G-25 tubes and stored at −40°.

Polyacrylamide gel electrophoresis under nondenaturing conditions and polyacrylamide slab gel electrophoresis in the presence of SDS were carried out according to published techniques [24, 25].

Immunodiffusion. Purified acetyl-CoA carboxylase from 9686 was used to prepare antisera, and immunodiffusion was done according to ref. [26].

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