Acetyl-Coenzyme A Carboxylase from Avocado (*Persea americana*) Plastids and Spinach (*Spinacia oleracea*) Chloroplasts

Sudesh B. MOHAN and Roy G. O. KEKWICK

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

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Preparations of acetyl-CoA carboxylase [acetyl-CoA–carbon-dioxide ligase (ADP-forming), EC 6.4.1.2] have been obtained from the plastids of avocado (*Persea americana*) fruit mesocarp and from spinach (*Spinacia oleracea*) chloroplasts. Both preparations required bovine serum albumin, HCO_3^- , citrate and glycerol for stabilization. The molecular weight of the avocado enzyme was about 6.5×10^5 on the basis of 1 mol of biotin/mol of enzyme, the behaviour of both enzymes on gel filtration being in accord with such a value. Removal of the stabilizing bovine serum albumin resulted in the loss of a biotin-containing fragment from the avocado enzyme. Citrate stabilized the enzyme at 10 mM and activated it optimally at 3.0 mM, effecting an approx. 2-fold increase in V_{max} . It is suggested that *in vivo* the enzyme may be located within the chloroplast lamellae.

The key significance of malonyl-CoA as an intermediate in the biosynthesis of fatty acids in tissues from all species is beyond dispute. It is therefore surprising that the nature of the enzyme responsible for the formation of this important substance, acetyl-CoA carboxylase [acetyl-CoA–carbon-dioxide ligase (ADP-forming), EC 6.4.1.2], in green leaves and its relation to possibly different forms of the same enzyme that may be present in non-photosynthetic plant tissue is not clear.

Whereas somewhat similar soluble preparations of the enzyme have been obtained from wheat (*Triticum* sp.)-germ (Hatch & Stumpf, 1961; Heinstein & Stumpf, 1969) and barley (*Hordeum* sp.) embryo (Brock & Kannangara, 1976), both of which contain an active macromolecular unit of particle weight about 6.2×10^5 daltons, the system carboxylating acetyl-CoA in green leaves seems to be quite different. Burton & Stumpf (1966) were indeed unable to detect acetyl-CoA carboxylase activity in preparations of lettuce (*Lactuca sativa*) chloroplasts actively synthesizing fatty acids. Although Kannangara & Stumpf (1972) were also unable to demonstrate acetyl-CoA carboxylase in disrupted spinach (*Spinacia oleracea*) chloroplasts, assays in which the activities of the soluble and particulate fractions of such disrupted chloroplasts were supplemented by those of the components of purified *Escherichia coli* acetyl-CoA carboxylase led

to the conclusion that the components of the spinach enzyme were distributed between the soluble and particulate phases of the chloroplasts, a sequence of carboxylation of a biotin-containing component of the lamella followed by discharge of the carboxy group to acetyl-CoA being catalysed by two enzyme components in the stroma.

The relation between acetyl-CoA carboxylase and fatty acid synthetase in the green plastids of the mesocarp of the avocado fruit appeared from previous studies in this laboratory (Weaire & Kekwick, 1975a,b) to be akin to that prevailing in mature chloroplasts. Thus the particulate system obtained from avocado mesocarp plastids by ultrasonic disintegration, which displayed a bicarbonate-dependent synthesis of fatty acids from acetate, was neither able to utilize exogenous malonyl-CoA nor was it susceptible to the inhibition by avidin characteristic of carboxylating systems dependent on biotin. From this it was inferred that any acetyl-CoA carboxylase present must be integrated in a membrane in a close association with the fatty acid synthetase system, impenetrable by malonyl-CoA and inaccessible to avidin.

It was therefore clearly essential to establish the existence of this enzyme, whose presence in the plastids had been inferred, and to investigate its relation to the apparently different systems of wheat and barley embryo on the one hand and leaf chloroplasts on the other.

The work reported in the present paper forms part

Abbreviation used: SDS, sodium dodecyl sulphate.

of the thesis submitted to the University of Birmingham for the Degree of Doctor of Philosophy by S. B. M.

Experimental

Materials

Avocado (*Persea americana*) fruits were obtained from local retailers and were used within 3–4 days. Spinach (*Spinacia oleracea*) was also purchased from local retailers and used immediately. *Lactobacillus plantarum* N.C.I.B. 6376 used for biological assay of biotin was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K.

Acetyl-CoA was prepared by the method of Simon & Shemin (1953) and its concentration determined by the difference in A_{232} of acetyl-CoA and of free CoA formed on hydrolysis (Stadtman, 1957). Ribulose biphosphate (barium salt) was obtained from Sigma and was converted into the sodium salt as described by Rabin & Trown (1964). $\text{NaH}^{14}\text{CO}_3$ was purchased from The Radiochemical Centre.

ATP (disodium salt), avidin, biotin, bovine serum albumin, dithiothreitol, *N*-acetylcysteine, polyvinylpyrrolidone (1-ethenyl-2-pyrrolidinone polymers), phenyl chloromercuribenzoate and phenylmethanesulphonyl fluoride, were obtained from Sigma. Di-isopropyl phosphorofluoridate was obtained from Koch-Light. CoA and rabbit muscle lactate dehydrogenase (EC 1.1.1.27) were purchased from Boehringer. Bacto-Yeast extract, Bactotryptose, Bactobiotin dehydrated assay medium and agar were obtained from Difco. Acrylamide, *NN*-dimethylenbisacrylamide, *NNN'*-tetramethylethylenediamine and riboflavin were obtained from Kodak. Sephadex and Sepharose were obtained from Pharmacia, and molecular-weight markers (range 53 000–265 000) for SDS/polyacrylamide-gel electrophoresis were obtained from BDH. All other chemicals were obtained from Fisons, BDH or Koch-Light, and were AnalaR grade.

Methods

Plastid preparation and homogenization. Avocado plastids were prepared by the procedure of Weaire & Kekwick (1975a); the grinding medium, however, was modified and contained 5 mM-sodium isoascorbate, 5 mM-sodium metabisulphite and 1 mM-sodium cyanide in addition to the 0.3 M-sucrose, 10 mM-KCl, 20 mM-Tris/HCl buffer, pH 7.2, 4 mM-*N*-acetylcysteine, 0.5 mM-EDTA and 0.7 mg of bovine serum albumin/ml of the original medium. Spinach chloroplasts were prepared in the same medium by the procedure of Walker (1964).

Both plastids and chloroplasts were homogenized at 4°C for 1 min at full speed in an Ato-Mix blender in a homogenizing medium that contained 0.1 M-potassium phosphate buffer, pH 7.3, 0.5 mM-EDTA, 1 mM-dithiothreitol, 5 mM-sodium isoascorbate, 5 mM-sodium metabisulphite, 1 mM-sodium cyanide, 10 mM-sodium citrate, 15 mM-potassium bicarbonate, 0.1% bovine serum albumin and 1% polyvinylpyrrolidone.

Enzyme assays. Acetyl-CoA carboxylase. This was assayed by measuring the rate of incorporation of $\text{H}^{14}\text{CO}_3^-$ into malonyl-CoA. From the investigation of the optimum assay conditions for the purified enzyme, the following standard assay was developed. The standard incubation mixture consisted of 60 mM-Tris/HCl, pH 7.5, 3 mM- Na_2ATP , 4 mM- MgCl_2 , 3 mM-trisodium citrate, 15 mM- $\text{NaH}^{14}\text{CO}_3$ ($1\mu\text{Ci}/\mu\text{mol}$), 0.2–0.4 mM-acetyl-CoA, 1.0 mg of bovine serum albumin, 1 mM-dithiothreitol and enzyme protein (about 40 μg) in a final volume of 1 ml. After 10 min at 37°C, the reaction was terminated by adding 0.2 ml of 6 M-HCl. Any precipitated protein was removed by centrifugation at 4000g for 10 min at room temperature. A portion of the supernatant was dried for 1 h at 85°C in a scintillation vial. Water (1 ml) and scintillator (10 ml; Patterson & Green, 1965) were then added and the acid-stable radioactivity measured in a Philips liquid-scintillation counter. Blanks without acetyl-CoA or enzyme were included in each series of assays and, with the exception of column fractions, all assays were duplicated.

Ribulose biphosphate carboxylase activity. This was determined by the $\text{H}^{14}\text{CO}_3^-$ -fixation assay described by Rabin & Trown (1964), in which ribulose biphosphate-dependent fixation of $\text{H}^{14}\text{CO}_3^-$ into acid-stable 3-phosphoglycerate is measured.

Units of enzyme activity. All enzyme activities are expressed as units (1 unit = 1 μmol of substrate converted into product/h under the standard conditions specified).

Standard buffer. The solution referred to as 'standard buffer' comprised 0.1 M-potassium phosphate buffer, pH 7.3, containing 0.5 mM-EDTA, 1 mM-dithiothreitol, 10 mM-trisodium citrate, 15 mM- NaHCO_3 , 0.1% bovine serum albumin and 15% (v/v) glycerol.

Protein determination. The protein content of crude enzyme preparation was determined by the biuret method of Gornall *et al.* (1949) after the removal of interfering pigments as described by Weaire & Kekwick (1975a). The protein content of the purified enzyme was measured by the Lowry method described by Geiger & Bessman (1972). The protein content of column eluates was assayed by measuring A_{280} .

Polyacrylamide-gel electrophoresis. Disc electro-

phoresis was carried out in polyacrylamide gels by the method of Davis (1964). The resolving gel contained 5% (w/v) acrylamide, the tracking dye was Bromophenol Blue, and a current of 3 mA per gel was applied for about 1½ h. Protein was stained with Coomassie Brilliant Blue [0.25% solution in methanol/acetic acid/water (45:9:46, by vol.)]. Background stain was removed by washing in a solution containing acetic acid/methanol/water, (15:18:167, by vol.).

SDS/polyacrylamide-gel electrophoresis. This was performed as described by Weber & Osborn (1969) in 7% (w/v) polyacrylamide. Enzyme samples to be used were eluted from unstained polyacrylamide gels obtained by electrophoresis in 5% gels as described above. Portions of these gels were incubated in 0.01 M-sodium phosphate buffer, pH 7.0, containing 1% SDS, or 1% SDS and 1% β -mercaptoethanol, at 37°C for several hours or at 100°C for 5 min.

After incubation the extracted protein was collected by centrifugation, dialysed against buffer containing 0.1% SDS, or 0.1% SDS and 0.1% β -mercaptoethanol, concentrated over poly(ethylene glycol) 6000 and applied to disc gels. After electrophoresis at 8 mA/gel for 4 h, gels were stained in Coomassie Brilliant Blue as described above. The molecular weights of the subunits were determined by comparing their mobilities with those of marker proteins of mol.wt. range 53 000–265 000 obtained from BDH.

Determination of biotin. The biotin content of the purified enzymes was determined by a microbiological turbidimetric assay with *Lactobacillus plantarum* (N.C.I.B 6376) as described by Gyorgy (1967), except that Bactobiotin dehydrated assay medium was used. Samples of enzyme were hydrolysed with 2 M-H₂SO₄ for 3 h at 120°C in sealed tubes to release biotin that was covalently bound. The hydrolysates were neutralized with 4 M-KOH,

diluted to a biotin concentration of about 0.5 ng/ml and stored at –20° until assayed. To release the biotin from acrylamide gels, proteins corresponding to the individual bands were eluted from unstained gels in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1% SDS by incubating at 37°C for several hours. The incubated samples were then centrifuged and the supernatants were extensively dialysed against 0.01 M-sodium phosphate buffer, pH 7.0 to remove SDS. This step was necessary because biotin could not be released in sufficient quantities by hydrolysing stained protein bands and because SDS inhibited bacterial growth. The extracted protein was then hydrolysed to release biotin as described above.

Immunodiffusion. Purified avocado enzyme obtained from the Sepharose-4B eluate to be used for antibody production was concentrated by pressure dialysis and the stabilizing bovine serum albumin removal by gel filtration through Sephadex G-200 previously equilibrated with the standard buffer from which bovine serum albumin had been omitted. Antisera were prepared and immunodiffusion analyses carried out by the procedure given by Gray & Kekwick (1974).

Results and Discussion

Some release of acetyl-CoA carboxylase from avocado plastids was obtained by osmotic rupture or by freezing and thawing. Although the specific activity of the osmotic lysate was nearly five times that obtained by freezing and thawing, Table 1 shows that about half of the enzyme remained in the membranous pellet. Homogenization of the plastids in an Ato-Mix blender gave a similar total yield of enzyme in the lysate, almost entirely in the soluble phase. Precipitation of the enzyme from this homogenate in the 20–40%-sat. (NH₄)₂SO₄ fraction consistently produced a nearly 3-fold increase in the

Table 1. *Distribution of acetyl-CoA carboxylase in fractions from lysed avocado plastids*

Plastids prepared as described by Weaire & Kekwick (1975a) were ruptured by (1) dispersion in 0.1 M-phosphate buffer, pH 7.3, containing 0.5 mM-EDTA, 3 mM-dithiothreitol and 60 mM-NaHCO₃, the pellet being resuspended and the fractions dissolved in this medium before assay with [1-¹⁴C]acetyl-CoA as substrate, or (2) by homogenization in an Ato-Mix blender in 0.1 M-phosphate buffer, pH 7.3, containing 0.5 mM-EDTA and 3 mM-dithiothreitol, the pellet being resuspended and the precipitate redissolved in the buffer before assay with NaH¹⁴CO₃ as substrate.

Fraction ...	Enzyme activity of homogenate 105 000 g supernatant (% of total)		
	105 000 g Pellet	0–20%-Satd.- (NH ₄) ₂ SO ₄ ppt.	20–40%-Satd.- (NH ₄) ₂ SO ₄ ppt.
Lysis procedure:			
(1) Osmotic shock	52.5	19.2	17.4
(2) Ato-Mix homogenization	6.5	2.6	370

total enzyme activity. The reason for this marked increase was not investigated. Attempts at further purification of the enzyme were frustrated by the extreme lability of the preparation, attributable in part to progressive inactivation by polyphenols produced during the homogenization and also to a possibly inherent instability of the enzyme. The problem was to some extent overcome by the inclusion of several additives in the homogenization and purification media.

Polyphenol oxidase activity in the homogenate was decreased by the addition of 5 mM-sodium isoascorbate, 5 mM-potassium metabisulphite and 1 mM-potassium cyanide in the grinding medium, and any remaining polyphenols were adsorbed on 1% polyvinylpyrrolidone in the homogenization buffer. The stability of the enzyme preparation was markedly improved by the inclusion of 15 mM- NaHCO_3 (cf. Kannangara & Stumpf, 1972), 0.1% bovine serum albumin (cf. Fritz & Hsu, 1966; Moss *et al.* (1972), 15% glycerol (successfully utilized by Miller & Levy, 1969) and 10 mM-sodium citrate in the purification buffer.

One of the main objects of the purification of acetyl-CoA carboxylase was to resolve it from the principal contaminating enzyme, ribulose biphosphate carboxylase, which comprises about 50% of leaf protein, but which is also present in avocado mesocarp; as it has (as will be seen below) a molecular weight somewhat similar to that of acetyl-CoA carboxylase, it tends to co-purify with this enzyme. Although the two enzymes could be partially resolved by density-gradient centrifugation, any possible increase in the ratio of acetyl-CoA carboxylase to ribulose biphosphate carboxylase activity was offset by the decay of the former enzyme during the procedure. As a result of the

almost capricious lability of the enzyme during the other preparative procedures attempted, the purification method described below, involving $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by gel filtration on Sepharose 4B at 4°C, was resorted to.

Acetyl-CoA carboxylase from avocado plastids

Disruption of the avocado mesocarp from 15–20 avocado fruits by the modified procedure of Weaire & Kekwick (1975a) (see under 'Methods') yielded a plastid preparation that was homogenized for 1 min in an Ato-Mix blender at full speed at 4°C in the homogenizing medium also described above. Removal of particulate material by centrifuging at 105 000g for 30 min yielded a supernatant that was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation at pH 7.3 and 4°C. The 20–40%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction was re-dissolved in the 'standard buffer' (see above), giving a fraction having nearly six times the total activity and almost 100 times the specific activity associated with only 6% of the protein of the parent homogenate supernatant (Table 2). Elution of this material from a column of Sepharose 4B (Fig. 1a) resulted in a partial resolution of the acetyl-CoA carboxylase from the ribulose biphosphate carboxylase. The fractions containing acetyl-CoA carboxylase uncontaminated with ribulose biphosphate carboxylase were combined to give a preparation (Sepharose 4B fraction 1, Table 2a) having 16.5 times the specific activity and accounting for 35% of the total acetyl-CoA carboxylase activity of the parent 20–40%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate.

Acetyl-CoA carboxylase from spinach chloroplasts

The preparative procedure used for avocado plastids also enabled the enzyme to be prepared from spinach chloroplasts. The 20–40%-satd.-

Table 2. *Preparation of acetyl-CoA carboxylase*
Abbreviation used: n.d., not detectable. For further details, see the text.

Fraction	Protein (mg)	Acetyl-CoA carboxylase		Ribulose biphosphate carboxylase	
		Total activity ($\mu\text{mol of HCO}_3^-$ fixed/h)	Specific activity ($\mu\text{mol of HCO}_3^-$ fixed/h per mg of protein)	Total activity	Specific activity
(a) Avocado plastids					
Plastid homogenate	3000	9.85	0.003	0.96	0.0003
20–40%-Satd. $(\text{NH}_4)_2\text{SO}_4$	188	56.5	0.305	0.89	0.003
Sepharose 4B					
Fraction I	4.0	20.0	5.03	n.d.	n.d.
Fraction II	5.2	18.2	3.64	0.78	0.15
(b) Spinach chloroplasts					
Chloroplast homogenate	4450	37.4	0.008	801	180
20–40%-Satd. $(\text{NH}_4)_2\text{SO}_4$	145.5	72.4	0.498	869	597
Sepharose 4B fraction	37.2	55.6	1.50	500	1344

Fig. 1. Separation of acetyl-CoA carboxylase and ribulose biphosphate carboxylase on Sepharose 4B (a) 20–40%-(NH₄)₂SO₄ fraction from disrupted avocado mesocarp plastids; (b) 20–40%-(NH₄)₂SO₄ fraction from disrupted spinach chloroplasts. For each experiment 140 mg of protein was applied in 10 ml of standard buffer (see under 'Methods') to a column (2.5 cm × 100 cm) of Sepharose 4B. The protein was eluted with the standard buffer and the enzyme activities were assayed as described under 'Methods'. ●, A₂₈₀; ▲, acetyl-CoA carboxylase; ■, ribulose biphosphate carboxylase.

(NH₄)₂SO₄ fraction applied to the column of Sepharose 4B was, as would be expected, more heavily contaminated with ribulose 1,5-bisphosphate carboxylase than the corresponding avocado plastid fraction. On elution, only the two leading fractions were free of this contamination (Fig. 1b). Table 2(b) shows the recoveries of activities obtained in this fractionation.

Identity of the product

The identity of the product obtained by the action of the avocado enzyme on acetyl-CoA and NaH¹⁴CO₃ was verified by the identification of the derived [¹⁴C]malonyl monohydroxamate. Mixtures containing five times the standard quantities of the enzyme-assay reagents in a total volume of 5.0 ml were incubated for 30 min at 37°C; an equal volume of neutral hydroxylamine solution (0.18 M) was then added and the mixture incubated for a further 30 min. Paper chromatography of the concentrated ethanol extract of the mixtures in the upper phase of butan-1-ol/acetic acid/water (4:1:4, by vol.) re-

vealed a single radioactive component coincident with authentic malonyl hydroxamate.

Homogeneity and biotin content of the enzyme

Electrophoresis of the purified avocado enzyme in 5% acrylamide gels revealed the presence of a single slow-moving component in addition to the albumin added for stabilization. This component contained biotin and was attributed to the enzyme. Antisera raised to the purified enzyme gave a single precipitin line on gel diffusion against the parent homogenate.

The biotin content of the enzyme was also indicated by its sensitivity to inhibition by avidin, a 90% inhibition being obtained when 2.0 μmol of avidin was included in the standard 2.0 ml incubation mixture. Preincubation of avidin (2.0 μmol) with biotin completely relieved the inhibition. Assay of the purified avocado enzyme showed it to contain 1 mol of biotin/653 kg of enzyme.

Subunit structure and dissociation of the avocado enzyme

Elution of the biotin-containing band obtained on polyacrylamide-gel electrophoresis gave material that, on further electrophoresis in polyacrylamide gels containing 1% SDS, was found to contain three subunits of mol.wts. 47 000, 57 000 and 120 000 respectively (Fig. 2). Electrophoresis in 1% SDS/polyacrylamide gels 'fortified' with 1% mercaptoethanol revealed the presence of four subunits of mol.wt. 47 000, 57 000, 72 000 and 120 000 respectively. Similar electrophoretic analyses of enzyme preparations obtained in the presence of the proteinase inhibitor phenylmethanesulphonyl fluoride lead to results indistinguishable from those obtained in the absence of the inhibitor.

The bovine serum albumin required to stabilize the enzyme could not be removed by ultrafiltration of the purified avocado enzyme through an Amicon M100A filter (permeable to substances of mol.wt. less than 100 000), although complete removal of bovine serum albumin from the buffer was effected by this procedure. The stabilizing bovine serum albumin was, however, successfully removed by gel filtration on Sephadex G-200 (Fig. 3a). The enzyme activity was eluted in the first protein peak; although only 4% of the applied activity was accounted for, all the biotin of the parent fraction was recovered therein. Assay of the biotin and protein contents of the fraction showed that 677 kg of protein would contain 1 mol of biotin, which, when taken with an elution volume similar to that of ribulose biphosphate carboxylase (mol.wt. 5.57×10^5 ; Paulsen & Lane, 1966) on Sepharose 4B shown by the enzyme (Fig. 1) and with the assumption that the preparation was essentially pure, indicates a molecular weight for avocado acetyl-CoA carboxylase of 6.77×10^5 . Electrophoresis of this fraction in 5%

Fig. 2. Electrophoresis of avocado acetyl-CoA carboxylase from the Sepharose 4B column

(a) Enzyme preparation dissociated and separated in gels containing 1% SDS; (b) enzyme preparation dissociated and separated in gels containing 1% SDS and 1% β -mercaptoethanol.

polyacrylamide gel (Fig. 3b) revealed, in addition to the presence of a large component barely penetrating the gel (band 1), the dissociation of a faster component (band 2), which contained all the applied biotin.

Optimal conditions for and stabilization of enzyme activity

Avocado acetyl-CoA carboxylase was markedly pH-sensitive between pH 6.0 and the optimum, pH 7.5 (Fig. 4a). The preparation also showed an unusual temperature-sensitivity, the activity at the optimum (37°C) being about ten times that at 20°C (Fig. 4b).

The avocado enzyme retained all its activity on storage in the standard buffer at -20°C for 2 months and 75% of that activity on storage in the standard buffer for 1 week. Removal of glycerol or bovine serum albumin from the standard buffer resulted in total loss of activity, whereas selective removal of either citrate or HCO_3^- or removal of both these ions resulted in losses of 60–70% of the original activity. The optimal concentration for stabilization by citrate or isocitrate was 10 mM, and

Fig. 3. (a) Removal of stabilizing bovine serum albumin from the purified avocado acetyl-CoA carboxylase (obtained from the Sepharose 4B column) by filtration on a Sephadex G-200 column by elution with albumin-free standard buffer containing 10 mM-citrate, and (b) electrophoresis of fractions I (left) and II (right) from the Sephadex G-200 eluate in 5% polyacrylamide gel

(a) The column dimensions were 2.5 cm $\times$ 100 cm. O, $A_{280}^{1\text{cm}}$; $\blacktriangle$, acetyl-CoA carboxylase activity. (b)

The hydrolysate from the region corresponding to band 2 of fraction I from six pooled gels was found to contain about 5.0 ng of biotin; no biotin could be detected in the other components of either fraction. For further details, see the text.

although such a citrate concentration inhibited the enzyme, that of isocitrate did not.

Solutions of the enzyme containing less than 20 μg of enzyme protein/ml lost activity on dilution with the standard buffer (Fig. 4c), this loss of activity being independent of the presence of bovine serum albumin in the buffer.

Inhibition of the avocado enzyme

Iodoacetamide (10 mM), CuSO_4 (1 mM) and phenyl

Fig. 4. Optimal conditions for avocado acetyl-CoA carboxylase activity

(a) Change of enzyme activity with pH; (b) relation between enzyme activity and temperature; (c) effect of protein concentration. The buffers used were: pH 4.0–5.5, sodium acetate/sodium dihydrogen phosphate; pH 6.0–7.0, sodium dihydrogen phosphate/sodium hydrogen phosphate; pH 7.5–9.5, Tris/sodium hydrogen phosphate.

chloromercuribenzoate all completely inhibited the avocado enzyme. A surprising inhibition by the serine-proteinase inhibitor di-isopropyl phosphorofluoridate was also observed, but 45% of this could be ascribed to the propan-2-ol solvent, and since there is no other evidence for the involvement of a serine in the activity of acetyl-CoA carboxylase, the source of the inhibition may have been an impurity in the di-isopropyl phosphorofluoridate such as was reported by Gould & Liener (1965) to inhibit ficin.

Kinetic characteristics

Both enzyme preparations required either Mg²⁺ or Mn²⁺ for activity and were activated by citrate or isocitrate. Activation by citrate showed a pronounced optimum at a concentration of 3.0 mM, concentrations in excess of 4.0 mM being inhibitory; activation by isocitrate was also maximal at 3.0 mM, but higher concentrations were not inhibitory. The roles of these two cations and two anions in the activation of the acetyl-CoA carboxylase were investigated.

Effect of Mg²⁺ and Mn²⁺

The dependence of the avocado carboxylase on MgCl₂ or MnCl₂ in the presence of constant ATP concentrations is seen in Fig. 5 to be sigmoidal. Calculation of the concentrations of the different ions in the incubation by the procedure of Storer and Cornish-Bowden (1976), by using the stability constants of Phillips *et al.* (1966) and O'Sullivan (1969), revealed that ATP⁴⁻ and ATPH³⁻ were the predominant forms of ATP at low MgCl₂ concentrations. The maximal concentrations of MgATP²⁻ coincided with the optimal concentration of MgCl₂ for carboxylase activity. From this it was inferred that MgATP²⁻ was a substrate and that the sigmoidal relation shown in Fig. 5 arose from

Fig. 5. Effect of various concentrations of Mg²⁺ (a) and Mn²⁺ (b) in the presence of different constant concentrations of ATP on the velocity of the avocado enzyme (concentration of acetyl-CoA, 0.4 mM; NaH¹⁴CO₃, 15 mM) ATP concentrations: ■, 1.1 mM; ○, 1.75 mM; ●, 3.5 mM.

inhibition by the relatively high proportion of ATP⁴⁻ and ATPH³⁻ prevailing at low concentrations of MgCl₂. Fig. 6 shows the result of assaying the enzyme at different ATP concentrations, each of which was 1 mM less than the simultaneously varied MgCl₂ concentration. This procedure gives both a constant concentration of Mg²⁺ (Cleland, 1967) and a constant proportion of ATP as MgATP²⁻ (Storer & Cornish-Bowden, 1976). When the concentrations of the different ionic species were again calculated by using the Storer–Cornish-Bowden (1976) procedure, it was found that maximal activity was obtained at 3.0 mM-MgCl₂, corresponding to a calculated concentration of 1.3 mM-MgATP²⁻. A similar sigmoidal relation between wheat-germ acetyl-CoA carboxylase activity and MgCl₂ concentration has been ascribed by Nielsen *et al.* (1979)

to a dual requirement of Mg^{2+} for the formation of $MgATP^{2-}$ and for enzyme activation. The maximum enzyme activity shown in Fig. 6 in the presence of a constant concentration of 0.1 mM-Mg^{2+} suggests that, if the avocado enzyme is similarly activated, it must be at a lower concentration of Mg^{2+} than 0.1 mM .

Fig. 6. Relation between avocado enzyme activity and ionic composition calculated by the method of Storer & Cornish-Bowden (1976), of the incubation medium at various concentrations of ATP with the Mg^{2+} concentration in 1 mM excess over the ATP concentration. Concentrations: citrate, 3.0 mM ; HCO_3^- , 15 mM ; acetyl-CoA, 0.4 mM . Curves: 1, ATP^{4-} ; 2, $ATPH^{3-}$; 3, $MgATP^{2-}$; 4, Mg^{2+} ; 5, cit^{3-} (cit=citrate); 6, magnesium citrate; 7, $MgHPO_4$; 8, $Hcit^{2-}$; 9, $MgHATP^-$. ■, acetyl-CoA carboxylase activity.

Effect of citrate and isocitrate on the kinetic constants

The kinetic constants K_m and V_{max} were determined for the action of the enzyme on HCO_3^- , acetyl-CoA and $MgATP^{2-}$ and on $MnATP^{2-}$, the

Fig. 7. Relation between enzyme activity and ATP concentration in the presence of related $MnCl_2$ concentrations. O, $MnCl_2$ concentration in 1 mM excess over ATP; □, $MnCl_2$ concentration equal to ATP; Δ, $MnCl_2$ concentration 1 mM less than that of ATP.

Table 3. Kinetic constants of acetyl-CoA carboxylase

Portions (3 ml) of the purified enzyme were desalted on columns of Sephadex G-25 by elution with the standard buffer from which HCO_3^- had been omitted, containing either 10 mM -citrate or -isocitrate so that the concentrations given below were obtained on dilution of the eluate in the incubation mixtures. The enzyme ($40\mu\text{g}$ of protein) was incubated at 37°C in a mixture in which the substrate concentrations, except for that being varied, were standard. Thus the apparent K_m values for acetyl-CoA and ATP were determined at 15.0 mM-HCO_3^- , those for HCO_3^- and ATP at 4.0 mM -acetyl-CoA, and those for acetyl-CoA and HCO_3^- at 3.0 mM -ATP. The concentration of Mg^{2+} was in 1.0 mM excess over that of ATP and that of Mn^{2+} , 2 mM . Apparent K_m values are in mM and V_{max} values are in μmol of HCO_3^- fixed/h per mg of protein. Abbreviation used: n.d., not determined.

		Substrate ... Acetyl CoA		HCO_3^-		ATP	
Mn^{2+} or Mg^{2+}	[Citrate] or [isocitrate]	Apparent K_m	V_{max}	Apparent K_m	V_{max}	Apparent K_m	V_{max}
Avocado							
Mg^{2+}	0	0.26	6.65	8.0	6.65	0.35	2.50
	3.0 mM -Citrate	0.26	11.1	8.0	11.1	0.30	11.1
	1.0 mM -Isocitrate	0.28	8.32	8.0	8.30	0.30	5.2
	3.0 mM -Isocitrate	0.30	11.1	8.0	11.1	0.30	11.1
Mn^{2+}	0	0.28	12.5	8.0	18.2	n.d.	n.d.
	3.0 mM -Citrate	0.28	16.7	8.0	23.6	n.d.	n.d.
	1.0 mM -Isocitrate	0.30	12.5	8.0	18.2	n.d.	n.d.
	3.0 mM -Isocitrate	0.28	16.7	8.0	23.6	n.d.	n.d.
Spinach							
Mg^{2+}	0	0.1	0.357	3.0	0.500	0.25	0.475
	3.0 mM -Citrate	0.1	0.625	3.0	0.625	0.35	0.550

concentration of one substrate being varied in the presence of constant concentrations of the other two. Whereas the concentration of MgATP^{2-} was optimal, that of acetyl-CoA was 0.4 mM and that of HCO_3^- 15 mM. The K_m values given in Table 3 are therefore apparent, and the values of $V_{\max}$ vary with the substrate. There was, however, no evidence of a dependence of the value of K_m for one substrate on the concentration of another. In view of the activation by citrate and isocitrate, determinations of apparent K_m and $V_{\max}$ values were made in the presence and absence of these ions. Stabilizing HCO_3^- and citrate were removed from the enzyme preparation before assay. Removal of HCO_3^- , necessary to avoid dilution of the $\text{NaH}^{14}\text{CO}_3$ substrate, resulted in a 70% loss of activity; removal of citrate resulted in no such immediate loss of activity. Care was taken, however, to complete the assay within 2–3 h of removal of the stabilizing ion.

It is clear from the results in Table 3 that the citrate or isocitrate had a negligible effect on the K_m values for the various substrates, but there was a perceptible increase in the values of $V_{\max}$ obtained with each substrate in the presence of the activator.

The kinetic characteristics of the two enzyme preparations were on the whole similar, but the avocado enzyme had a slightly higher K_m than the spinach enzyme for both acetyl-CoA and NaHCO_3 . Both enzyme preparations were saturated with about 0.6 mM-acetyl-CoA; the presence of 3 mM-citrate raised the concentration of acetyl-CoA required for saturation of the avocado enzyme to 1.6 mM, no similar effect being observed on the spinach enzyme. Again, in the presence of Mn^{2+} but in the absence of citrate, a concentration of 1 mM-acetyl-CoA inhibited the avocado enzyme but not the spinach enzyme. Whereas the spinach enzyme was saturated with 10 mM- NaHCO_3 in both the presence and the absence of 3.0 mM citrate, the avocado enzyme required a concentration of 20 mM- NaHCO_3 for saturation in the absence of citrate and was not saturated by this concentration of NaHCO_3 in the presence of 3.0 mM-citrate.

Conclusions

It appears from the results presented that avocado mesocarp plastids, spinach chloroplasts, wheat-germ and barley embryo contain essentially similar forms of acetyl-CoA carboxylase. The reported particle weight of the enzyme ranges between 6.1×10^5 and 6.5×10^5 daltons in the four preparations, such units each containing one molecule of biotin. Those kinetic properties of the wheat-germ enzyme that have been reported resemble those obtained for the avocado and spinach enzymes. It is not clear, however, whether citrate activation is a property shared with wheat-germ and barley-embryo enzymes by avocado plastid and spinach chloroplast enzymes.

Burton & Stumpf (1966) 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. Hein-stein & Stumpf (1969) on the other hand reported that the wheat-germ enzyme was not affected by 4 mM-isocitrate and that higher concentrations were inhibitory.

All the preparations of plant acetyl-CoA carboxylase described have been found to dissociate, but the nature of the subunits obtained appears to differ both with the preparations investigated and the techniques applied. Thus the biotin-containing fragment yielded when the avocado enzyme was subjected to electrophoresis in polyacrylamide gels in the absence of albumin (Fig. 3b) is clearly much smaller than the 7.3S biotin-containing component obtained from the wheat-germ enzyme on ultracentrifugation in a sucrose gradient by Hein-stein & Stumpf (1969). The three components obtained on electrophoresis of the avocado enzyme in SDS/polyacrylamide gels were, however, much larger than the three apparently analogous fragments having mol.wts. of 41 000, 32 000 and 21 000 respectively obtained by Brock & Kannangara (1976) on electrophoresis of the barley-embryo enzyme in polyacrylamide gels containing 8 M-urea.

It seems clear, both from the data presented in Table 1 (showing that about half the avocado enzyme remained in the particulate fraction after osmotic rupture of the plastids) and from the data obtained previously by Weaire & Kekwick (1975b), that the avocado enzyme is bound to a plastid membrane. Rupture of this membrane to release the enzyme may result in a consequent loss of conformational restraints, leading to a labile configuration requiring both albumin and citrate for stabilization and also susceptible to activation by lower concentrations of citrate. The multiplicity of precipitin lines obtained when the crude plastid homogenate was allowed to diffuse against the antiserum to the purified enzyme may also indicate the presence of membrane portions of different sizes adhering to the enzyme. The results obtained with the spinach chloroplast acetyl-CoA carboxylase, taken with the observations of Kannangara & Stumpf (1972), are also consistent with a membrane origin of that enzyme. Although Kannangara & Jensen (1975) have considered the barley-embryo enzyme to be soluble, the results of Brock & Kannangara (1976) show that, on ultracentrifugation of the parent homogenate, the enzyme activity is also associated with particles. It is therefore possible that further investigations will show that not only is acetyl-CoA carboxylase associated with fatty acid synthetase in the chloroplast lamellae and the membranes of green plastids of avocado mesocarp,

but that a similar association in the membranes of plastids occurs in non-photosynthetic tissues.

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