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Spinach Leaf Acetyl-Coenzyme A Synthetase: Purification and Characterization[1]

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ABSTRACT

Acetyl-coenzyme A (CoA) synthetase was purified 364-fold from leaves of spinach (*Spinacia oleracea* L.) using ammonium sulfate fractionation followed by ion exchange, dye-ligand, and gel permeation chromatography. The final specific activity was 2.77 units per milligram protein. The average M_r value of the native enzyme was about 73,000. The Michaelis constants determined for Mg-ATP, acetate, and coenzyme A were 150, 57, and 5 micromolar, respectively. The purified enzyme was sensitive to substrate inhibition by CoA with an apparent K_i for CoA of 700 micromolar. The enzyme was specific for acetate; other short and long chain fatty acids were ineffective as substrates. Several intermediates and end products of fatty acid synthesis were examined as potential inhibitors of acetyl-CoA synthetase activity, but none of the compounds tested significantly inhibited acetyl-CoA synthetase activity *in vitro*. The properties of the purified enzyme support the postulated role of acetyl-CoA synthetase as a primary source of chloroplast acetyl-CoA.

Acetyl-CoA occupies a central position in the integrated metabolism of the plant cell. This two carbon acetyl unit is an essential metabolite entering the citric acid cycle and is a fundamental precursor for fatty acids and isoprenoids, the building blocks for membrane lipids and polyisoprenoids, *e.g.* phytohormones, quinones, carotenoids, and the phytol tail of Chl.

One mechanism for generating acetyl-CoA found in both procaryotic and eucaryotic organisms (15) is the acetate activating enzyme, acetyl-CoA synthetase (acetate:CoA ligase, EC 6.2.1.1). This enzyme catalyzes the reversible reaction: acetate + CoA + ATP $\rightleftharpoons$ acetyl-CoA + AMP + PPi. Acetyl-CoA synthetase has been isolated and extensively purified from a number of animal tissues (15) and yeast (7).

In leaves, acetyl-CoA synthetase is localized exclusively in the chloroplast (12), where its proposed function is to provide acetyl-CoA for anabolic reactions. Acetyl-CoA synthetase's role as a sole contributor to chloroplast acetyl-CoA, however, has been questioned because of the identification of the chloroplast isozyme of the PDC[3] (3, 26). The chloroplast PDC could potentially provide a more direct route for acetyl-CoA synthesis from photosynthetic products (18). Studies examining the incorporation of different radiolabeled substrates showed exogenous acetate to be the preferred substrate for fatty acid biosynthesis in isolated spinach (*Spinacia oleracea* L.) chloroplasts (21, 22), but pyruvate was preferred by *Sinapis alba* chloroplasts (14). A recent report by Springer and Heise (24) showed with isolated spinach chloroplasts that radiolabeled acetate at physiological concentrations preferentially labeled fatty acids whereas radiolabeled pyruvate showed preference for products of prenyl metabolism. Heintz *et al.* (9) reported that in developing plastids of barley leaves pyruvate was a precursor (via chloroplast PDC) for isoprenoids and fatty acids, but that as the tissue matured the chloroplast PDC declined as a precursor of C_2 units. Heintz *et al.* (9) further showed that when acetate was provided to both developing and mature barley chloroplasts it was the primary source of C_2 units for fatty acid biosynthesis.

Significant levels of acetate have been measured in leaf tissue from several plant species (12, 27) and in a spinach leaves; 15 to 20% of the total cellular acetate coincided with the chloroplast fraction (27). Taken together, this evidence indicates that acetyl-CoA synthetase is at least partially responsible for chloroplast acetyl-CoA formation in most plant tissues.

Despite the potential importance of acetyl-CoA synthetase in chloroplast acetyl-CoA metabolism, no attempt has been made to purify this enzyme from photosynthetic tissue. Millerd and Bonner (17) reported some properties of the enzyme using ammonium sulfate precipitated enzyme from spinach leaf extracts. This enriched spinach enzyme showed rather broad substrate specificity, with butyrate and succinate being 84 and 52%, respectively, as active as acetate, but showed no activity with propionate as substrate. Millerd and Bonner (17) attributed the substrate nonspecificity to the presence of several activating enzymes. Young and Anderson (32) identified a short chain fatty acyl-CoA synthetase in extracts of germinated seeds of *Pinus radiata* which had a greater affinity for butyrate than for acetate, but no butyryl-CoA synthetase activity has been reported for leaf tissue. In contrast, acetyl-CoA synthetase purified from several different sources (4, 7, 10, 29) activated acetate and propionate but not butyrate. Therefore, it was our intent to investigate whether acetyl-CoA synthetase isolated from spinach leaves is indeed an acetyl-

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[3] Abbreviations: PDC, pyruvate dehydrogenase complex; ACP, acyl carrier protein; FPLC, fast protein liquid chromatography.

CoA synthetase and not a short chain fatty acyl-CoA synthetase capable of using exogenous acetate as substrate; develop a protocol for at least partially purifying spinach leaf acetyl-CoA synthetase for further kinetic analysis; and characterize the biochemical properties of the purified enzyme. A preliminary report describing some of the data in this paper was presented previously (33).

MATERIALS AND METHODS

Chemicals

DEAE-Sephacel and the FPLC Superose 12 were purchased from Pharmacia, Inc. Red A dye matrix gel was from Amicon Corp. Nucleoside triphosphates except ATP, thiols, thioesters except acetyl-CoA, and Sephadex G-100 were obtained from Sigma Chemical Co. ATP, acetyl-CoA, and LiCoA were from P-L Biochemicals. ACP was from Cal Biochem. Palmitoyl-ACP was a generous gift from Dr. John Ohlrogge, Michigan State University. [1-^{14}C]acetate and [1-^{14}C]oleate were purchased from Amersham. [2-^{14}C]propionate was obtained from DuPont-New England Nuclear. Scintiverse E was purchased from Fisher.

Assay of Acetyl-CoA Synthetase

Acetyl-CoA synthetase activity was measured by the radioassay as described by Roughan *et al.* (22) with some modifications. The 250 μL reaction mixture contained 50 mM Hepes-KOH (pH 8.0), 5 mM $MgCl_2$, 2 mM ATP, 0.05 mM CoA, and 0.5 mM DTT. The reaction were initiated with 1 mM [1-^{14}C]acetate (20 μCi/μM) after a 2 min preincubation at 30°C. The aliquots from the reaction mixture were quenched at 1, 2, and 3 min by applying 20 μL to a 2.5 cm^2 piece of Whatman No. 1 filter paper and placing the filter paper into a beaker containing 400 mL of 3:1 ethanol:diethyl ether (v/v) with 0.25% (v/v) TCA at 4°C. The filter paper stayed in the first wash no longer than 90 min. Longer wash times resulted in significant loss of [^{14}C]acetyl-CoA bound to the filter paper. The filter paper was periodically and gently agitated with a stir rod to ensure complete removal of the unesterified [^{14}C]acetate. The filter papers were subsequently washed with an additional 400 mL of the ethanol-diethyl ether mixture (5 min) followed by a 200 mL diethyl ether wash (5 min). After the final wash, the filter papers were air-dried, placed in scintillation vials with 5 mL of Scintiverse E, and counted using an LKB 1214 Rackbeta scintillation counter. The radioactivity appearing in acetyl-CoA was plotted as a function of time and the slope taken as the initial velocity.

Purification of Acetyl-CoA Synthetase

Spinach (*Spinacia oleracea* L.) leaves purchased from a local market were washed, midribs removed, and 450 g homogenized with a Brinkman polytron in the presence of 1350 mL of extraction medium. The extraction medium contained 100 mM Tris (pH 7.8), 5 mM 2-mercaptoethanol, 5 μM leupeptin, 1 mM benzamidine, 1 mM ϵ-aminocaproic acid, and 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth (Cal Biochem) and centrifuged at 14,600*g* for 30 min to remove cell debris. The supernatant was brought to 40% saturation by addition of solid ammonium sulfate. After constant stirring for 30 min at 4°C, the mixture was centrifuged at 14,600*g* for 30 min. The resulting supernatant was brought to 60% saturation by addition of ammonium sulfate and equilibrated for 30 min at 4°C. Following centrifugation at 14,600*g*, the 40 to 60% ammonium sulfate pellet was dissolved in a minimal volume of buffer A, which contained 20 mM Tris, pH 7.8, 5 mM 2-mercaptoethanol, and 20% (v/v) glycerol. The redissolved pellet was concentrated/desalted with buffer A using Amicon protein concentrator with a PM10 filter and then applied to a DEAE-Sephacel column (27 cm × 2.5 cm) equilibrated in buffer A. The column was washed with buffer A until the A_{280} absorbance had returned to 0.0. The remaining protein bound to the column was eluted with a linear gradient of 0 to 0.5 M KCl. The active acetyl-CoA synthetase fractions were pooled and directly applied to a Red A dye-ligand column (12 cm × 2.5 cm) equilibrated in buffer A. The column was washed with buffer A followed by 500 mM KCl in buffer A. The A_{280} returned to 0 before each subsequent step was initiated. Acetyl-CoA synthetase was eluted with 1.0 M KCl in buffer A. The active fractions were pooled, concentrated with an Amicon protein concentrator fitted with a PM10 filter, and sucrose added to bring the sucrose concentration to 5%. The concentrated protein solution was applied to a Sephadex G-100 column (97 cm × 2.5 cm) equilibrated in buffer A and the column developed with buffer A. The active fractions collected from the column were pooled, glycerol added to a final concentration of 40%, and stored at −20°C until use. This final preparation was used as the purified acetyl-CoA synthetase. The entire purification procedure was carried out at 0 to 4°C.

Substrate Kinetic Analysis

The Michaelis constants of the respective substrates of acetyl-CoA synthetase were determined by varying one substrate at fixed saturating concentrations of the other two substrates. The data collected were fitted to the equation $v = V_{max} \times A/(K + A)$ using the Cleland least squares method (5).

Evaluation of Metabolic Effectors

The metabolites were dissolved in 50 mM Hepes, pH 8.0, except for the hydrophobic compounds, which were dissolved in chloroform. An aliquot of the hydrophobic compounds was pipetted into the reaction vial, the chloroform evaporated with N_2 gas, the hydrophobic compound redissolved in the reaction mixture, and vortexed vigorously. All the metabolic effectors tested were preincubated with the enzyme in the reaction mixture for 2 min before initiating the reaction by addition of [^{14}C]acetate.

Protein Determination

Protein content was estimated by the method of Bradford (1) using BSA as standard.

RESULTS

Purification of Spinach Leaf Acetyl-CoA Synthetase

Purification of acetyl-CoA synthetase from spinach leaf extracts utilized ammonium sulfate fractionation to concentrate protein and remove Chl from the extract. Typically, 60 to 100% of the total acetyl-CoA synthetase activity was recovered in the 40 to 60% ammonium sulfate fraction with a one- to two-fold enrichment. Following desalting, the ammonium sulfate fraction was applied to a DEAE-Sephacel column, with a typical elution profile presented in Figure 1. Only a single peak of acetyl-CoA synthetase activity eluted from the column at approximately 0.2 M KCl with a four- to fivefold enrichment.

Red A dye-ligand chromatography was an especially effective purification step for the spinach enzyme (Fig. 2). Most of the total protein eluted in either the buffer wash or with 500 mM KCl. Acetyl-CoA synthetase was eluted with 1 M KCl and resulted in a 38-fold purification for this single step. The active fractions pooled from Red A column were concentrated and further purified by Sephadex G-100 gel filtration. The results of a typical purification are summarized in Table I. The pooled enzyme obtained from Sephadex G-100 column gave a final specific activity of 2.77 units/mg protein and reflected a 364-fold purification with an overall recovery from crude extract of 11%. It was this final preparation which was used for the subsequent characterization and substrate kinetic analyses of spinach acetyl-CoA synthetase.

The spinach acetyl-CoA synthetase purified 364-fold was not homogenous. Electrophoresis of the enzyme purified through Sephadex G-100 on 10% nondenaturing gels revealed four major and three minor protein bands (data not shown). A single peak of acetyl-CoA synthetase was eluted from a 0.5 cm gel slice which corresponded to two unresolved protein bands.

Stability

Acetyl-CoA synthetase was found to be unstable in the absence of glycerol. Preliminary studies showed the enzyme purified through ammonium sulfate fractionation and FPLC

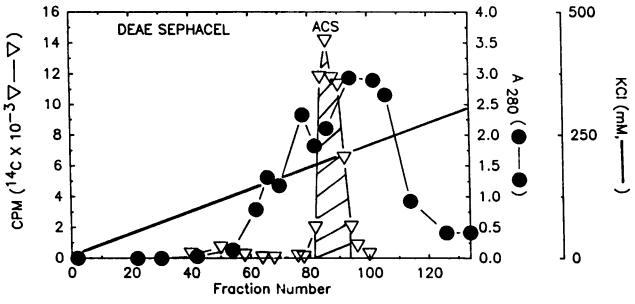

Figure 1. DEAE-Sephacel chromatography of spinach leaf acetyl-CoA synthetase. The DEAE-Sephacel column was equilibrated in buffer A as described in "Materials and Methods." Spinach leaf acetyl-CoA synthetase was eluted from the column with a linear 0 to 500 mM KCl gradient. Fractions of 5.3 mL were collected. The hatched area indicates fractions pooled for further purification.

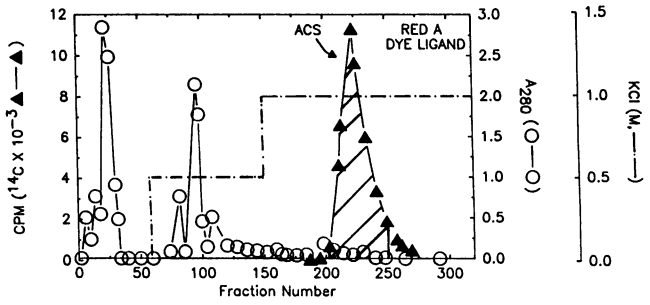

Figure 2. Elution profile of spinach leaf acetyl-CoA synthetase from a Red A dye-ligand column. The column was washed with buffer A followed with 0.5 M KCl in buffer A before eluting acetyl-CoA synthetase with 1 M KCl containing buffer. Each fraction contained 2.7 mL. Hatched area indicates fractions pooled for further purification.

Superose 12 gel filtration had 95% loss of activity at 4°C and 43% loss of activity at −20°C after 17 d storage. Therefore, all columns maintained at 4°C were equilibrated and eluted with buffers containing 20% glycerol. The purified acetyl-CoA synthetase was also adjusted to 40% glycerol before storing at −20°C. Under these conditions the purified enzyme was stable with essentially no loss of activity after storage at −20°C for 3 weeks and was still active after storage for 18 months.

Properties of Acetyl-CoA Synthetase

The M_r value of acetyl-CoA synthetase estimated by using a calibrated Sephadex G-100 column was 69,000, while 77,000 was estimated using a calibrated FPLC Superose 12 column (data not shown).

The activity of partially purified acetyl-CoA synthetase was measured over the pH range 6.4 to 9.2. Acetyl-CoA synthetase activity showed a broad pH optimum of 7.5 to 8.5 with 50% of maximum activity observed at pH 6.6.

Acetyl-CoA synthetase activity increased with assay temperature between 0 and 40°C (Fig. 3A) in the presence of 50 μM CoA, but 500 μM was necessary to stabilize the enzyme to 50°C. Complete loss of activity was observed if the purified enzyme was preincubated at 60°C for 30 min prior to assaying at 30°C. Arrhenius plots were linear between 0 and 50°C in the presence of 500 μM CoA and between 0 and 40°C at 50 μM CoA (Fig. 3B). The ratio of activity at 40°C to that at 30°C was 1.64 and 1.46 and the calculated activation energy was

Table I. *Purification Summary of Acetyl-CoA Synthetase from Leaves of Spinacia oleracea*

Fraction	Total Protein	Specific Activity	Total Activity	Yield	Enrichment
	mg	*units/mg*	*units*	%	*-fold*
Crude	3377	0.008	25.66	100	
40 to 60% $(NH_4)_2SO_4$	1860	0.008	15.25	59	1.1
DEAE-Sephacel	339	0.043	11.61	45	4.5
Red A	5	1.30	5.92	23	171.0
Sephadex G-100	1	2.77	2.77	11	364.0

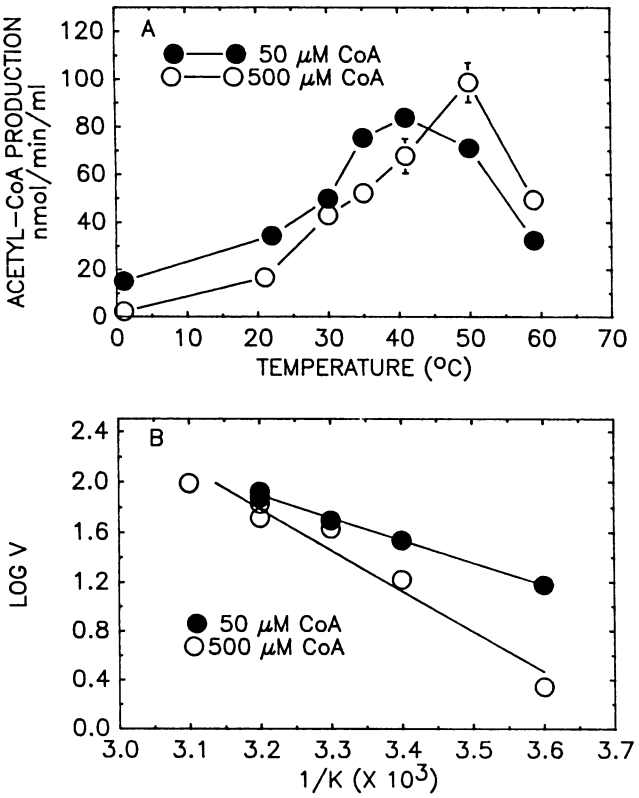

Figure 3. A, The temperature optimum for purified acetyl-CoA synthetase at 50 μM CoA and 500 μM CoA and B, Arrhenius plot of the data. The standard reaction mixture was preincubated at the appropriate temperature for 5 min before initiating the reaction with enzyme. The temperature of the water bath was measured before and after each assay.

8.19 and 14.5 kcal/M at 50 μM CoA and 500 μM CoA, respectively.

Thiol Requirement

The purified enzyme had an absolute requirement for thiol protection with maximal activity observed at a DTT concentration of 1 mM. In the absence of DTT the enzyme was inactive. Other reduced mono and dithiol compounds present at 1.0 mM substituted for DTT but oxidized thiols were ineffective in activating acetyl-CoA synthetase (Table II).

DTT was not required in the reaction mixture to keep CoA reduced. DTT could be removed from the reaction mixture and the purified enzyme remained active during the assay period if acetyl-CoA synthetase was preincubated in DTT prior to adding the enzyme to the reaction mixture. DTT in the preincubation mixture could be reduced to a concentration as low as 0.1 mM before a significant loss of activity was observed.

Substrate Specificity

The extreme high cost of radiolabeled substrates dictated that we examine substrate specificity indirectly. Substrates of known acyl-CoA synthetases were thus tested as possible competitive inhibitors of the purified enzyme (Table III). Nonradioactive substrates were added to the assay mixture containing about twice K_m concentrations of [1-^{14}C]acetate and saturating levels of Mg-ATP and CoA. Neither succinate, malonate, butyrate, or oleate competed significantly with [1-^{14}C]acetate at equal or 5× concentration. Propionate was somewhat competitive, inhibiting acetyl-CoA synthetase 40% at a 10-fold higher concentration (1 mM).

To establish that the inhibition by propionate was due to competition for potential substrate and not a nonspecific inhibition, the formation of propionyl-CoA was directly measured and compared to acetyl-CoA formation. Direct measurement of propionyl-CoA synthesis from 0.5 mM sodium [2-^{14}C]propionate gave a specific activity of 0.1 μM/min·mg protein compared to 1.0 μM/min·mg protein for acetate at the same concentration. No activity was observed when 0.5 mM [1-^{14}C]oleic acid was substituted for acetate.

Of the nucleotide triphosphates examined, only ATP supported acetyl-CoA formation. GTP, UTP, XTP, CTP, and ITP at concentrations of 2.0 mM did not substitute for ATP. Acetyl-CoA synthetase activity was reduced 57% when dATP replaced ATP.

Substrate Kinetics

The purified spinach enzyme showed hyperbolic saturation curves for Mg-ATP and acetate (Fig. 4 A, B). The apparent K_m values for Mg-ATP and acetate estimated from Lineweaver-Burk plots were 150 and 57 μM, respectively. In contrast to the other substrates, saturation curves for CoA deviated from normal Michaelis-Menten kinetics (Fig. 5A). Maximum activity was obtained at 83 μM CoA and activity declined at higher CoA concentrations. At 500 μM CoA, acetyl-CoA synthetase activity was inhibited 64%. The apparent K_m for CoA using points on the saturation curve free of inhibition was 5 μM and the V_{max} was 2.1 unit/mg protein.

To estimate an inhibition constant for CoA, a theoretical CoA saturation curve was constructed using the Michaelis-Menten equation and K_m and V_{max} values determined from the noninhibitory portion of the experimental saturation curve. Assuming that the theoretical curve represents the activity where no inhibition occurs, a percentage of inhibition was calculated. An apparent K_i value for CoA was determined from a plot of reciprocal percentage of inhibition *versus*

Table II. *Effect of Thiols on Spinach Acetyl-CoA Synthetase Activity*

Thiol	Concentration	Relative Activity
	mM	%
DTT (control)[a]	1.00	100
−DTT	0.00	4
+Oxidized DTT	1.00	3
+DTE	1.00	105
+Glutathione	1.00	96
+Oxidized glutathione	1.00	47
+2-Mercaptoethanol	1.00	88
+2-Mercaptoethanol	10.00	95

[a] The control activity was 2.9 units/mg protein.

Table III. *Effect of Potential Competing Substrates on the Activation of Acetate*

Treatment[a]	Concentration	Rate Relative to Control[b]
	mM	
Succinate	0.10	1.09
	0.50	0.95
	1.00	1.02
Malonate	0.10	1.01
	0.50	1.03
	1.00	1.04
Butyrate	0.10	0.79
	0.50	0.81
	1.00	0.83
Propionate	0.10	0.94
	0.50	0.76
	1.00	0.59
Oleate	0.05	1.17
	0.10	1.03
	0.50	1.04
	1.00	1.09
Acetate (unlabeled)	0.05	0.70
	0.10	0.57
	0.50	0.25
	1.00	0.15

[a] The reaction mixture also contained 5.0 mM $MgCl_2$, 2.0 mM ATP, 0.5 mM CoA, 0.5 mM DTT, and 100 μM [1-^{14}C]acetate (20 μCi/μM). The reaction was initiated with enzyme. [b] Control rate = 1.0.

reciprocal CoA concentration (Fig. 5B). Using this approach, the apparent K_i value estimated for CoA was approximately 700 μM.

Substrate inhibition was most pronounced between pH 7.5 and 9.0, the same region of the pH profile found optimum for the purified enzyme (Fig. 6). As the pH became more acidic, the CoA inhibition was reduced. At pH 6.5, the percentage of inhibition was 26% compared to 43% at pH 8.0. Maximum inhibition at the same pH found optimal for acetyl-CoA synthetase activity suggested that CoA-enzyme and enzyme-inhibitor complexes had similar ionic interactions.

Regulation by Metabolic Effectors

The acetyl-CoA produced by acetyl-CoA synthetase is a substrate for fatty acid biosynthesis. Therefore, several fatty acid compounds were tested as possible feedback inhibitors. Palmitate (50 μM), oleate (50 μM), or their CoA thioester derivatives (100 μM) were without effect on acetyl-CoA synthetase activity. Palmitoyl-ACP, ACP, and malonyl-CoA (50–100 μM), likewise, had no effect on activity.

DISCUSSION

This report presents the most extensive purification and characterization of an acetyl-CoA synthetase from plant tissue and the only purification report describing the enzyme from leaf tissue. Huang and Stumpf (10) resolved five forms of the potato tuber acetyl-CoA synthetase on DEAE-cellulose which were not interconvertible under different but undefined experimental conditions. The different forms of the enzyme from potato tubers had similar properties (*i.e.* molecular size, pH optimum, substrate specificity, response to inhibition by AMP) but differed in their apparent charge. Using a similar anion exchange matrix, DEAE-Sephacel, only a single activity peak was observed for the spinach leaf enzyme, suggesting that under our experimental conditions the spinach enzyme existed as a single charged species.

Several of the dye-ligand columns (*i.e.* Green A, Blue A, Red A) from Amicon Corp. were successful in binding spinach acetyl-CoA synthetase, with the enzyme having a particularly strong affinity for the Red A dye-ligand column. The tight binding of acetyl-CoA synthetase to the Red A dye-ligand column was advantageous in that, in addition to its effectiveness as a purification step, it allowed acetyl-CoA synthetase active fractions from the DEAE-Sephacel column to be directly applied to this column without a prior desalting step.

The estimation of the M_r by two different calibrated gel filtration systems gave an average M_r value of 73,000 for the spinach acetyl-CoA synthetase. The M_r value of the potato tuber acetyl-CoA synthetase has been reported to be 59,500 (10). Acetyl-CoA synthetase purified from ox heart mitochondria has a reported M_r value of 57,000 (16), whereas the

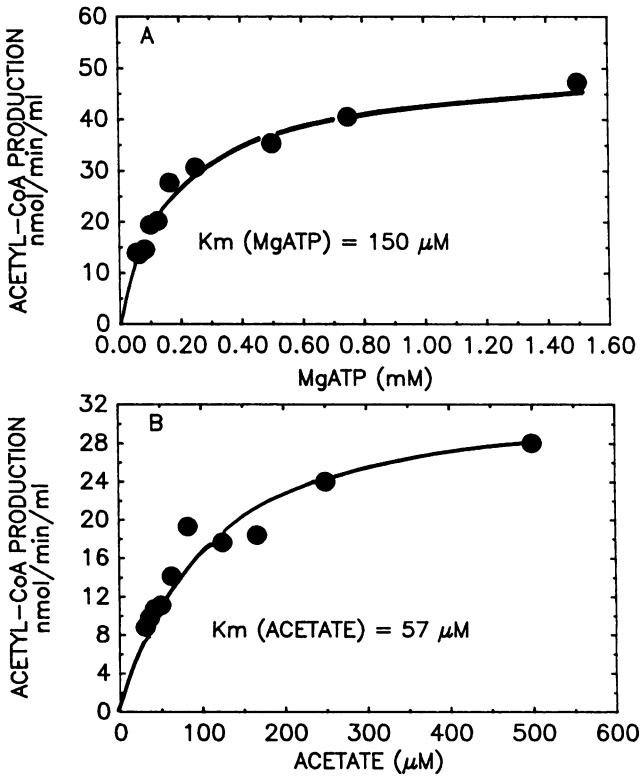

Figure 4. Effect of substrate concentration on acetyl-CoA synthetase activity when either A, Mg-ATP or B, acetate was the variable substrate. When Mg-ATP was the variable substrate, the acetate and CoA concentration were held constant at 1 and 0.5 mM, respectively. The ATP and CoA concentration were held constant at 2 and 0.5 mM, respectively, when acetate was the variable substrate. In both plots, the Mg^{2+} was fixed at a constant excess concentration of 3 mM.

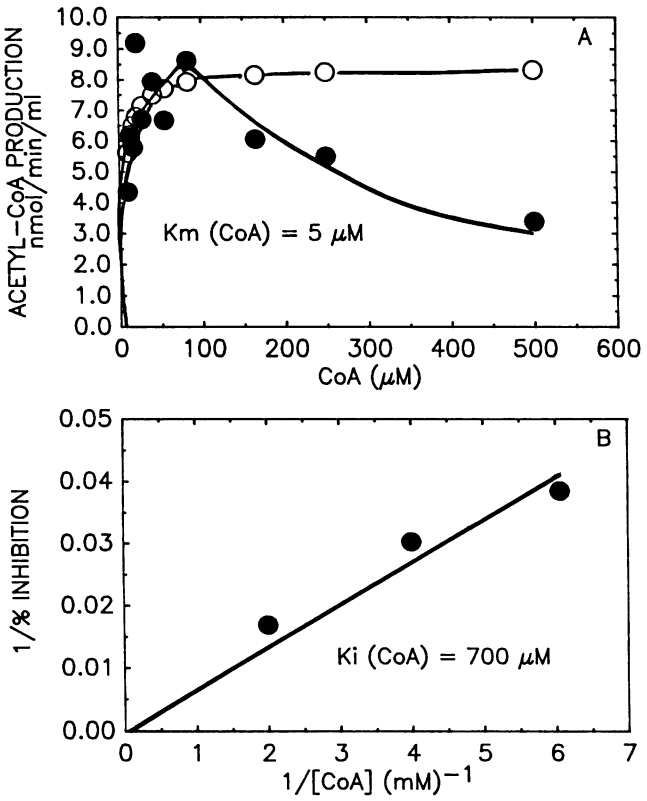

Figure 5. A, The CoA saturation curve for purified acetyl-CoA synthetase. B, Lineweaver Burk plot of the data points from the inhibitory portion of the experimental saturation curve. The closed circles represent the experimental saturation curve. Acetate, ATP, and Mg^{2+} were held constant at 1.0, 2.0, and 5.0 mM, respectively. The open circles represent the theoretical saturation curve calculated from the Michaelis-Menten equation using K_m and V_{max} values estimated from the noninhibitory portion of the experimental curve.

reported M_r value of acetyl-CoA synthetase from yeast was 151,000 (7).

An early report by Millerd and Bonner (17) showed acetyl-CoA synthetase from an ammonium sulfate precipitate of spinach leaf extract to activate acetate and butyrate with nearly equal efficiency. In contrast, both substrate competition experiments and direct measurement of acyl-CoA formation using representative short and long chain fatty acids as substrates established acetate as the primary substrate for the purified spinach enzyme. This supports Millerd and Bonner's (17) original postulate that more than one activating enzyme was in their enzyme preparation. Of the substrates tested, only propionate substituted for acetate. However, with propionate as substrate the activity was 10-fold lower than for acetate at an equivalent concentration ($9 \times K_m$ [acetate]), suggesting that propionate had a much lower affinity for the enzyme than acetate. This observation was consistent with the higher K_m values for propionate of 11 mM and 10 mM compared to the K_m values for acetate of 0.79 mM and 0.28 mM reported for the bovine heart mitochondria (4) and yeast (7) enzyme, respectively. The specificity of the purified spinach enzyme for acetate established the purified enzyme as an acetyl-CoA synthetase and not a short chain fatty acyl-CoA synthetase capable of using exogenous acetate as substrate. This is consistent with the postulated role for acetyl-CoA synthetase in spinach leaves as being a primary source of chloroplast acetyl-CoA.

The spinach enzyme was specific for ATP. A similar nucleotide triphosphate specificity requirement has previously been reported for acetyl-CoA synthetase from a number of sources (4, 10, 15) and is consistent with acetyl-CoA synthetase forming an acyladenylate intermediate (28) as part of its reaction mechanism.

The spinach enzyme required reduced thiols for activity. Huang and Stumpf (10) and Webster (31) have similarly reported that thiols stabilize the activity of the potato tuber and bovine heart mitochondria enzyme. Londesborough *et al.* (16) identified four thiol residues on ox heart mitochondria acetyl-CoA synthetase which react readily with *p*-hydroxymercuricbenzoate, causing an immediate inhibition of acetyl-CoA synthetase activity reversible only by CoA and 2-mercaptoethanol. They concluded from these studies that the reduced thiol residues were required to preserve the enzyme in the proper conformation for catalysis.

Both dithiol and monothiol compounds fulfilled the requirement for a thiol to activate acetyl-CoA synthetase. This suggested that the light modulation of spinach acetyl-CoA synthetase observed by Sauer and Heise (23) did not involve a ferredoxin-thioredoxin type control mechanism. Both phosphoribulokinase and alkaline fructose-1,6-bisphosphate phosphatase, enzymes regulated by the ferridoxin/thioredoxin system, showed preferential activation by dithiol compounds (2, 13). Additional activation of acetyl-CoA synthetase by dithiols might be overlooked because of the nonenzymatic side reaction between acetyl-CoA and DTT to form acetyl-DTT (25). Because the filter paper assay for acetyl-CoA synthetase (11) is based on the interaction of the adenine nucleotide moiety of CoA with the filter paper (but the radiolabel is present on

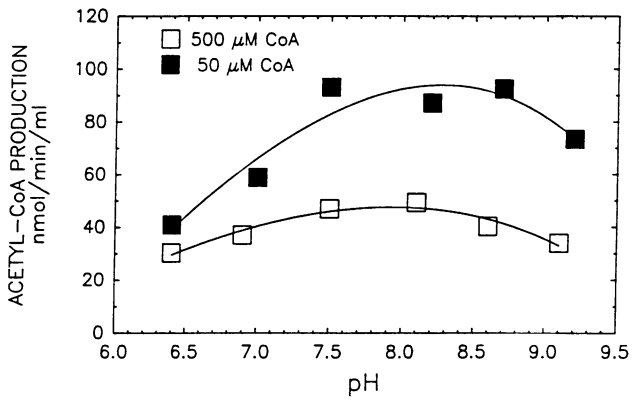

Figure 6. The pH dependency of purified acetyl-CoA synthetase at two different CoA concentrations. The buffer consisted of 50 mM Mes, 50 mM Hepes, and 50 mM Bicine adjusted to the respective pH with 10 N KOH. The reaction was initiated with enzyme and the pH determined at completion of assay using nonradioactive samples assayed concurrently. The nonradioactive samples were quenched by boiling rather than by the procedure outlined in the "Materials and Methods" section.

the first carbon of the acetate moiety), the formation of acetyl-DTT would prevent radiolabel detection of increased acetyl-CoA production. This argument seems unlikely because increasing 2-mercaptoethanol 10-fold (a thiol which is not as reactive with CoA thioesters [25]) did not significantly affect acetyl-CoA synthetase activity. Glutathione at concentrations comparable to that observed in the chloroplast (4 mM) under both light and dark conditions (6, 8) would be sufficient to keep the enzyme reduced and active *in vivo*.

The Michaelis constants of the spinach enzyme were 57 μM (acetate), 5 μM (CoA), and 150 μM (Mg-ATP). By comparison, the K_m values for the potato tuber enzyme were lower, 37 and 26 μM for Mg-ATP and acetate, respectively (10), while the K_m values for Mg-ATP and acetate from other organisms were higher than those for plants. The bovine heart mitochondria (29) and yeast (7) enzymes showed apparent K_m values for acetate of 0.8 and 0.28 mM, respectively, while K_m values for Mg-ATP were 0.9 and 1.2 mM for the same enzymes, respectively. The apparent K_m for CoA of the purified spinach enzyme was lower than for the bovine heart mitochondria enzyme (0.4 mM; 29) but was in a similar range as the yeast (35 μM; 7) and potato tuber enzyme (21 μM; 10).

The spinach enzyme is inhibited by CoA concentrations greater than 83 μM with an estimated K_i of 700 μM. Such sensitivity to substrate inhibition by CoA has not previously been reported for acetyl-CoA synthetase from any organism. Although Campagnari and Webster (4) found that the bovine heart mitochondria enzyme was inhibited by high concentrations of ATP (*i.e.* >4 mM), this inhibition can be attributed to the limited Mg^{2+} in their reaction mixture (30). CoA inhibition of the spinach acetyl-CoA synthetase is probably not physiologically significant but rather a consequence of the large excess of CoA (about 100-fold K_m) we, initially, and others (22, 23) have typically had present in the reaction mixture. On the other hand, it is important to be aware of such an inhibition, as high concentrations of CoA in the reaction mixture can lead to erroneous conclusions about the properties of the enzyme, as we will show with respect to the enzyme's free Mg^{2+} requirement in a subsequent paper.

In a leaf cell, the chloroplast is the exclusive site of fatty acid synthesis (20). Since previous studies indicate that acetate can be a primary precursor of chloroplast fatty acids, several intermediates and end products of fatty acid synthesis were tested for their effect on acetyl-CoA synthetase activity. However, none of the compounds tested affected the spinach enzyme *in vitro*. Although inhibition by oleate was reported for the rat hepatic acetyl-CoA synthetase (19), a similar inhibition by oleate was not observed with the spinach enzyme at concentrations ranging between 0.050 and 1.0 mM. On the other hand, the chloroplast PDC which is a potential alternative source of chloroplast acetyl-CoA was inhibited 58% by 50 μM oleate, suggesting that PDC may be sensitive to negative feedback control by oleate (3). Although both the study on pea chloroplast PDC and the study presented here on spinach leaf acetyl-CoA synthetase are limited to properties of the enzyme *in vitro*, the differences in sensitivity of these two enzymes to oleate suggests that these two mechanisms for synthesizing acetyl-CoA may be subjected to different cellular control mechanisms. The relative contribution of these two enzymes to the chloroplast acetyl-CoA pool may depend on the metabolic state of the chloroplast.

In summary, acetyl-CoA synthetase isolated and partially purified from spinach leaves has properties similar to those previously described for acetyl-CoA synthetase from other organisms and, in particular, the purified enzyme was quite specific for acetate. The properties of the spinach leaf enzyme are compatible with its proposed role in providing acetyl-CoA for anabolic processes in the chloroplast.

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