Purification, characterization, and ontogeny of acetyl-CoA carboxylase isozyme of chick embryo brain

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Abstract Acetyl-CoA carboxylase catalyzes the first committed step in the synthesis of fatty acids. Because fatty acids are required during myelination in the developing brain, it was proposed that the level of acetyl-CoA carboxylase may be highest in embryonic brain. The presence of acetyl-CoA carboxylase activity was detected in chick embryo brain. Its activity varied with age, showing a peak in the 17–18-day-old embryo and decreasing thereafter. The enzyme, affinity-purified from 18-day-old chick embryo brain, appeared as a major protein band on polyacrylamide electrophoresis gels in the presence of sodium dodecyl sulfate (M_r 265,000), indistinguishable from the 265 kDa isozyme of liver acetyl-CoA carboxylase. It had significant activity (Sp act = 1.1 μmol/min per mg protein) in the absence of citrate. There was a maximum stimulation of only 25% in the presence of citrate. Dephosphorylation using [acetyl-CoA carboxylase] phosphatase 2 did not result in activation of the enzyme. Palmitoyl-CoA (0.1 mM) and malonyl-CoA (1 mM) inhibited the activity to 95% and 71%, respectively. Palmitoylcarnitine, however, did not show significant inhibition. The enzyme was inhibited (>95%) by avidin; however, avidin did not show significant inhibition in the presence of excess biotin. The enzyme was also inhibited (>90%) by antibodies against liver acetyl-CoA carboxylase. An immunoblot or avidin-blot detected only one protein band (M_r 265,000) in preparations from chick embryo brain or adult liver. These observations suggest that acetyl-CoA carboxylase is present in embryonic brain and that the enzyme appears to be similar to the 265 kDa isozyme of liver. —**Thampy, K. G., and A. G. Koshy.** Purification, characterization, and ontogeny of acetyl-CoA carboxylase isozyme of chick embryo brain. *J. Lipid Res.* 1991. **32:** 1667–1673.

Supplementary key words avidin • biotin • citrate • liver acetyl-CoA carboxylase

Malonyl-coenzyme A has only one known metabolic role in animal tissues, in its incorporation into fatty acids. For this reason the formation of malonyl-CoA is considered as the first committed step in the synthesis of fatty acids. In animal tissues malonyl-CoA is formed from acetyl-CoA, catalyzed by acetyl-CoA carboxylase (EC 6.4.1.2), a classical cytosolic enzyme (1, 2). The enzyme is enriched in lipogenic tissues such as liver, adipose, and lactating mammary gland. The liver enzyme is a polymer ($M_r \geq 10$ million) composed of ≥40 identical subunits (3, 4) of mol wt 265,000 (5, 6), each subunit containing one biotin prosthetic group, two catalytic activities, namely biotin carboxylase and transcarboxylase, and the allosteric site for citrate.

A new isozyme of acetyl-CoA carboxylase, first described by Thampy (7), was shown to be enriched in heart, a tissue with negligible fatty acid synthesis. This isozyme is immunologically distinct from the 265 kDa isozyme of liver and has a higher subunit molecular weight (280,000). This isozyme may be present in muscle and brown adipose tissue (8). The specific role for this 280 kDa isozyme is not known. In order to understand the role of this isozyme, the enzyme in brain was studied since this tissue is not known to be ketogenic.

Previous studies could not detect acetyl-CoA carboxylase in brain (8). However, de novo fatty acid synthesis is known to take place in the brain. The synthesis of fatty acids follows a developmental pattern with highest activity observed in the developing brain (9, 10). The activity soon decreases and remains very low throughout adulthood. Since precursor fatty acids are required for the synthesis of myelin lipids and since myelination is an important event in the developing central nervous system the enzymes of fatty acid synthesis may be most active during this stage. Because myelinating activity is highest in the developing brain it was proposed that enzymes of fatty acid synthesis may be highest in the developing brain. Therefore, developing brain was chosen to study acetyl-CoA carboxylase. For the reason that the brain is not

Abbreviations: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

known to oxidize fatty acids, malonyl-CoA may serve the role of an intermediate in fatty acid synthesis only, and not as a regulatory molecule as in tissues that oxidize fatty acids. Developing brain was also found to be suitable to test the hypothesis that the 265 kDa and not the 280 kDa isozyme of acetyl-CoA carboxylase is present in lipogenic tissues that are inactive in fatty acid oxidation.

MATERIALS AND METHODS

Materials

The reagents for immunoblotting were obtained from Pierce Chemical Co. The sources of all other reagents and chemicals were mentioned previously (3, 11).

Methods

The identification and characterization of acetyl-CoA carboxylase isozyme involved purification of the enzyme from embryonic brain using avidin-affinity chromatography. The characterization of the enzyme involved enzyme assays, inhibitor studies, and immunologic comparison with acetyl-CoA carboxylase of rat and chick liver and rat heart.

Protein determination

The protein was determined by the bicinchoninic acid method according to the manufacturer's (Pierce) recommendations using bovine serum albumin as standard.

Carboxylase assay

The assay measured incorporation of [^{14}C]bicarbonate into malonyl-CoA (12). Each assay tube contained crude protein preparations or up to 1.0 μg of affinity-purified enzyme in a final volume of 0.15 ml. One unit of activity is defined as 1 μmol of malonyl-CoA formed per min at 37°C. The specific activity is defined as units/mg protein.

Purification of acetyl-CoA carboxylase

Brain was obtained from 18-day-old chick embryos and kept frozen at −70°C until used. In order to purify acetyl-CoA carboxylase, the tissue (160 g) was thawed and homogenized in a blender with 8 volumes of column buffer (100 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 0.5 M NaCl, 5% glycerol) containing 5 mg/l each of aprotinin, leupeptin, and antitrypsin and 5 mM benzamidine. All subsequent operations were carried out at 4°C. The homogenate was centrifuged at 30,000 *g* for 20 min and the resulting supernatant was fractionated by ammonium sulfate precipitation. The 0–30% ammonium sulfate pellet was dissolved in a minimum volume of column buffer and purified using avidin-Sepharose chromatography (12).

Preparation of rat liver [acetyl-CoA carboxylase]phosphatase 2

This was carried out using a modification of a published procedure (3, 12). The modification involved DEAE fractionation of the 5% polyethylene glycol supernatant (12) before ammonium sulfate fractionation. This method was less time-consuming than the initial ammonium sulfate fractionation, where separation of the viscous polyethylene glycol made the recovery of the precipitated protein tedious. Briefly, the 5% polyethylene glycol supernatant was diluted 4-fold using 10 mM Tris-HCl, pH 7.5. This was passed through DEAE-Bio-Gel (pre-equilibrated with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) in a fritted funnel. The gel was then washed with 10 volumes of 10 mM Tris-HCl, pH 7.5, containing 50 nM NaCl and 1 mM EDTA. The protein was eluted by raising the salt concentration to 0.25 M. The eluate was fractionated by precipitation using ammonium sulfate to 50% saturation. The precipitated protein was dissolved in a minimum volume of column buffer and kept frozen until used.

Activation of carboxylase using [acetyl-CoA carboxylase]phosphatase 2

The procedure was exactly as described (12) with the indicated modification (3). Briefly, purified acetyl-CoA carboxylase was preincubated for 20 min at 37°C with rat liver [acetyl-CoA carboxylase]phosphatase 2 in the presence of $MnCl_2$. After preincubation, the enzyme was assayed for carboxylase activity in the absence or presence of 10 mM citrate.

Purificaton of rat and chicken liver acetyl-CoA carboxylase

Rat liver acetyl-CoA carboxylase was purified from unfrozen livers with the indicated modifications (3, 12). In order to prepare chicken liver enzyme, two adult chickens were fasted for 3 days and re-fed for 2 days with a low-fat, high carbohydrate diet before being killed. The livers were quickly removed and acetyl-CoA carboxylase was isolated by avidin-affinity chromatography (12). The purified chicken liver enzyme had a specific activity of 0.3 and 5.2 U/mg in the absence and presence of 10 mM citrate, respectively. The purified rat liver enzyme had a specific activity of 0.6 and 3.3 U/mg in the absence and presence of 10 mM citrate, respectively.

Immobilization of chicken liver acetyl-CoA carboxylase

To 35 ml (packed volume) cyanogen bromide-activated Sepharose (13) 8 mg of chicken liver acetyl-CoA carboxylase in 50 mM potassium phosphate, pH 7.5, was added and equilibrated overnight at 4°C. Subsequently the gel was filtered. After blocking unreacted sites with aminoethanol, the gel was equilibrated with a solution

containing 150 mM NaCl and 10 mM potassium phosphate, pH 7.0.

Affinity purification of antibodies specific to 265 and 280 kDa isozymes of acetyl-CoA carboxylase

Rabbit antiserum to rat liver acetyl-CoA carboxylase was obtained and fractionated by ammonium sulfate precipitation (50% saturation). The ammonium sulfate fraction was dissolved in PBS and dialyzed overnight against PBS. The clarified solution was equilibrated with immobilized chicken liver acetyl-CoA carboxylase for 48 h. Subsequently the gel was filtered and the filtrate was separately collected. The gel was washed with PBS until A_{280} of the washings reached <0.005. The antibodies were then eluted by 0.1 M sodium acetate, pH 3.4, containing 0.4 M NaCl. Four-ml fractions were collected into tubes containing 1.0 ml of 1 M phosphate buffer, pH 8.0. Fractions with $A_{280}>0.05$ were collected, pooled, and kept frozen at $-70°C$ until used.

The antigen-affinity column was re-equilibrated with PBS and the filtrate from above was reapplied. After equilibration for 48 h at 4°C, the gel was filtered and the filtrate was collected as before. The filtrate was again applied to the regenerated antigen-affinity column and after equilibration for 48 h the filtrate was collected. The filtrate did not contain significant antibodies against the 265 kDa isozyme of acetyl-CoA carboxylase but was enriched in antibodies against the 280 kDa isozyme.

Electrophoretic blotting

The protein samples were subjected to electrophoresis on 5% polyacrylamide gels in the presence of SDS and then transferred to nitrocellulose according to Towbin, Staehelin, and Gordon (14). The method of detection followed the published procedure (14) except that albumin was replaced by a 5% solution of non-fat dry milk powder.

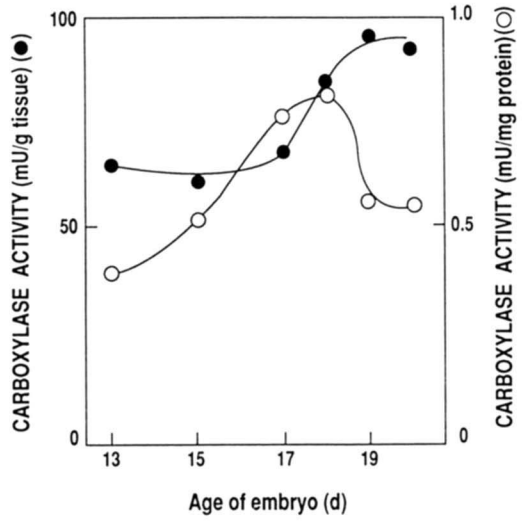

Fig. 1. Acetyl-CoA carboxylase activity in developing chick embryo brain. Brain from 4–5 chick embryos was homogenized in 3–4 vol of Column Buffer. The supernatant obtained after centrifugation at 12,000 *g* for 15 min was assayed for acetyl-CoA carboxylase exactly as described under Materials and Methods except that citrate was added to 10 mM. The pellet had negligible activity. The activities are given in mU/g tissue or mg total brain protein.

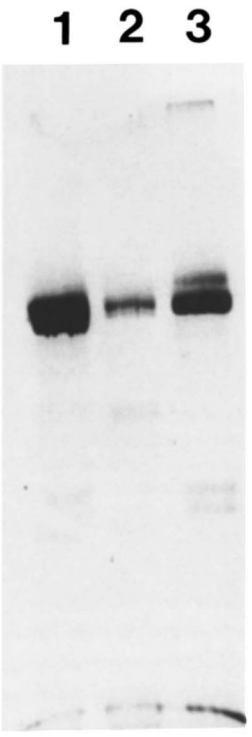

Fig. 2. Polyacrylamide gel electrophoresis in the presence of SDS. Purified acetyl-CoA carboxylase was subjected to electrophoresis on 5% polyacrylamide gels in the presence of SDS (22). The protein was visualized by silver staining. Lane 1, chicken liver acetyl-CoA carboxylase; lane 2, chicken brain acetyl-CoA carboxylase; lane 3, rat liver acetyl-CoA carboxylase.

RESULTS

Detection of acetyl-CoA carboxylase in developing brain

The activity of acetyl-CoA carboxylase in crude preparations from brain of 13-day-old chick embryo was low (**Fig. 1**) and found to increase with embryonic age. By day 17–18, there was a doubling of specific activity. However, the specific activity decreased thereafter. The total activity (per g wet tissue) was low on days 13–15 and increased 35% on days 19–20. The activity in the crude preparations required the presence of citrate, with only 20–24% activity observed in the absence of citrate.

Purity of brain acetyl-CoA carboxylase

As the specific activity of acetyl-CoA carboxylase was found to be highest in the 18-day-old embryo, brain from these embryos was used as a source for purification. The method of purification was rapid and involved ammoni-

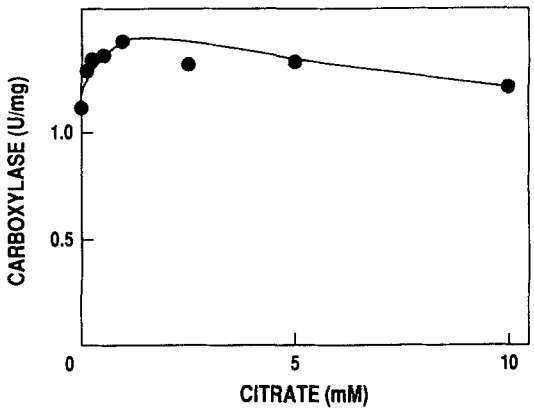

Fig. 3. Citrate dependence of acetyl-CoA carboxylase of chick embryo brain. Purified preparation of acetyl-CoA carboxylase from chick embryo brain was assayed for carboxylase activity exactly as described under Materials and Methods except that citrate was varied as indicated.

um sulfate fractionation and avidin-Sepharose chromatography. The purified enzyme preparation appeared as a major protein band (M_r 265,000) when analyzed by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (**Fig. 2**). The chicken or rat liver preparation contained a major protein band (M_r 265,000). The electrophoretic mobility of brain enzyme was indistinguishable from that of the liver acetyl-CoA carboxylase. The rat liver preparation also contained a minor protein band (M_r 280,000), while both chicken liver and brain preparations were free of this higher molecular weight species. The subunit molecular weight of brain enzyme was lower than that of the latter protein species. A minor protein, migrating slightly faster than the 265 kDa protein band was also seen in brain preparation.

Citrate dependence of acetyl-CoA carboxylase

The acetyl-CoA carboxylase activity in crude preparations from embryonic brain demonstrated citrate dependence. However, the purified preparation had significant activity (1.1 U/mg) when assayed in the absence of citrate (**Fig. 3**). The activity, under the same conditions of assay, was higher than that of chicken liver (0.3 U/mg) or rat liver enzyme (0.6 U/mg). Addition of citrate to chick brain enzyme resulted in a maximum of only 25% stimulation of activity. The specific activity of the brain enzyme in the presence of 10 mM citrate (1.4 U/mg) was lower than that of both rat liver (3.3 U/mg) and chicken liver enzyme (5.2 U/mg) under the same conditions of assay.

Incubation of brain enzyme with rat liver [acetyl-CoA carboxylase]phosphatase 2 resulted in no significant activation of carboxylase observed in the absence or presence of 10 mM citrate. Rat liver enzyme, under the same conditions, was activated by the phosphatase to 3.7 U/mg, assayed in the absence of citrate, in agreement with published results (12). The chicken liver enzyme was also activated by the phosphatase, however, the activation was much less significant (0.9 U/mg, assayed in the absence of citrate).

Effect of antibodies

Upon addition of polyclonal antibodies specific to rat liver acetyl-CoA carboxylase, the carboxylase activity in preparations from embryonic brain was almost completely inhibited in a dose-dependent manner (**Fig. 4**). The specific binding of the affinity-purified antibodies to rat and chicken liver and chicken brain carboxylase is shown in the immunoblot (**Fig. 5**). These antibodies were purified on immobilized chicken liver acetyl-CoA carboxylase (which contained only the 265 kDa species and no detectable 280 kDa species.) These antibodies are specific to the 265 kDa species without cross-reactivity with the 280 kDa species (7). The results indicate that enzyme preparations from chicken liver, brain, and rat liver were stained by this method and thus share antigenic characteristics. It is to be noted that the 265 kDa and not the 280 kDa species in rat liver preparation was stained by this method. Antibodies specific to 280 kDa isozyme specifically stained the 280 kDa species in rat heart and liver preparation (not shown). Since the antibodies against the 265 kDa isozyme of acetyl-CoA carboxylase specifically bind and inhibit brain acetyl-CoA carboxylase, it is suggested that the brain enzyme is immunolo-

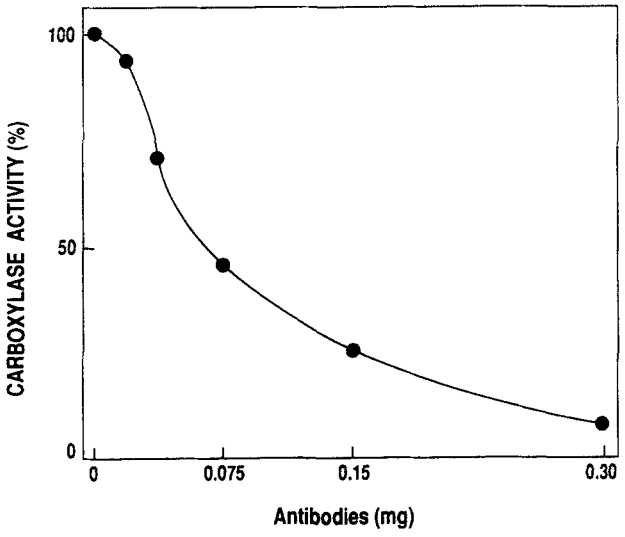

Fig. 4. Effect of antibodies on carboxylase activity. Purified acetyl-CoA carboxylase (2.3 μg) was mixed with the indicated amount of antibodies (ammonium sulfate fraction) specific to rat liver acetyl-CoA carboxylase in a final volume of 0.05 ml of phosphate-buffered saline, containing 0.75 mg/ml bovine serum albumin. This was incubated for 1 h at 0°C followed by 15 min at 37°C and then assayed for acetyl-CoA carboxylase in the presence of 10 mM citrate exactly as described under Materials and Methods.

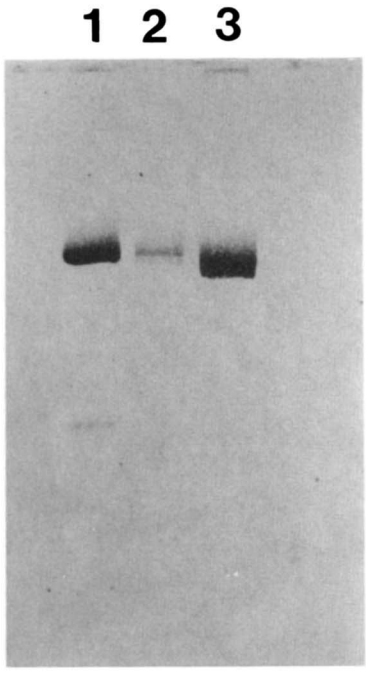

Fig. 5. Immunoblot. Acetyl-CoA carboxylase was subjected to electrophoresis on 5% polyacrylamide gels in the presence of SDS. Subsequently the proteins were electrophoretically transferred to nitrocellulose membrane and then probed with affinity-purified antibodies to 265 kDa isozyme of rat liver acetyl-CoA carboxylase (7). Lane 1, chicken liver acetyl-CoA carboxylase; lane 2, chicken brain acetyl-CoA carboxylase; lane 3, rat liver acetyl-CoA carboxylase.

gically similar to the 265 kDa isozyme. A protein band with electrophoretic mobility slightly faster than the 265 kDa species that was observed in Fig. 2 was also observed in the immunoblot (Fig. 5) with similar relative staining intensity.

Avidin blot

The binding of avidin to carboxylase was demonstrated in a Western blot using covalently linked avidin-peroxidase (**Fig. 6**). The preparations from liver and brain were stained by this technique. The enzyme preparation from brain showed the presence of a major band with molecular weight of 265,000. The preparation from liver also gave a major protein band with subunit molecular weight of 265,000. This indicates that both the brain and liver enzyme contained biotin. The presence of other biotin-containing proteins, such as pyruvate carboxylase or propionyl-CoA carboxylase, was not observed in these preparations.

Effect of inhibitors

The carboxylase activity in preparations from embryonic brain could be completely blocked by addition of avidin, an egg white protein that specifically binds biotin. The inhibition due to avidin could be reversed by addition of excess biotin (**Table 1**). This is characteristic of biotin-dependent carboxylation reactions. Malonyl-CoA, the product of carboxylase reaction, as well as palmitoyl-CoA inhibited the carboxylase activity (Table 1), quite characteristic of acetyl-CoA carboxylase from other tissues (7, 15). The inhibition by palmitoyl-CoA may be specific and not related to its detergent properties since palmitoylcarnitine, at the same concentration, did not show significant inhibition of carboxylase.

DISCUSSION

The results presented here indicate that acetyl-CoA carboxylase is present in the embryonic brain and its activity follows a developmental pattern with peak activity observed in the 17- to 18-day-old embryo. The observed peak activity (1.5 mU/mg supernatant protein) was comparable to that reported for forebrain (1.7 mU/mg supernatant protein) and lower than that for the brain stem (3.1 mU/mg supernatant protein) in developing rat brain (16). Beyond 18 days of embryonic age the specific activity of acetyl-CoA carboxylase decreases. In the adult the level of acetyl-CoA carboxylase is below the limit of detection. The peak activity of acetyl-CoA carboxylase in the 17- to 18-day-old embryo indicates high synthesis of fatty acids at this developmental stage presumably for incorporation into myelin lipids. In the adult brain, with much lower

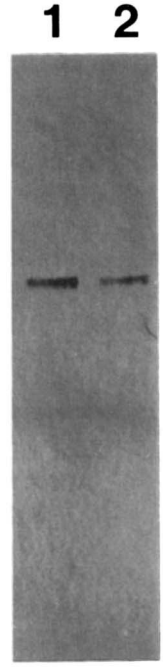

Fig. 6. Avidin-blot. Purified chicken liver acetyl-CoA carboxylase (lane 1) and chick brain acetyl-CoA carboxylase (lane 2) were subjected to electrophoresis on 5% polyacrylamide gels in the presence of SDS. The proteins in the gel were transferred to nitrocellulose and then probed with avidin-peroxidase as described under Materials and Methods.

TABLE 1. Effect of various compounds on the activity of brain acetyl-CoA carboxylase

Compounds Tested[a]	Conc.	% Activity
None		100
Malonyl-CoA	1.0 mM	29
Palmitoyl-CoA	0.1 mM	5
Palmitoylcarnitine	0.1 mM	86
Avidin[a]	0.15 nmol	2
Avidin + biotin[b]		83

[a]Purified preparations of acetyl-CoA carboxylase of brain were assayed in the presence of 10 mM citrate exactly as described in Materials and Methods except that the indicated concentration of malonyl-CoA or palmitoyl-CoA was included in the assay.

[b]When avidin or biotin was tested, the indicated amount was preincubated at 0°C for 30 min with 9 μg of acetyl-CoA carboxylase in 50 mM HEPES, pH 7.5, in a final volume of 0.1 ml. Subsequently, aliquots were withdrawn and assayed for acetyl-CoA carboxylase in the presence of 10 mM citrate, exactly as described in Materials and Methods.

myelin lipid synthesis, significant levels of acetyl-CoA carboxylase may not be present.

Recent studies indicated the presence of acetyl-CoA carboxylase activity in developing brain (16, 17). The present study identified this activity as authentic acetyl-CoA carboxylase. This was shown by actual isolation and purification of the activity from embryonic brain. The inhibition of the purified enzyme by palmitoyl-CoA, malonyl-CoA, antibodies against rat liver acetyl-CoA carboxylase, and avidin suggested that the activity is due to authentic acetyl-CoA carboxylase.

The isolated enzyme from embryonic brain was citrate-independent, similar to the enzyme in liver of fasted/refed rats (4). Acetyl-CoA carboxylase of animal tissues was once thought to have an absolute requirement of citrate for activity (18, 19). However, recent studies have shown that such citrate-dependence is due to the highly phosphorylated state of the enzyme (2–4, 12, 20). Upon in vitro dephosphorylation using [acetyl-CoA carboxylase]phosphatase 2, or by blocking phosphorylation during its isolation, an active citrate-independent form of acetyl-CoA carboxylase has been isolated (3, 12). The citrate requirement of the activity observed in crude preparatons of embryonic brain may be due to phosphorylation(s) taking place under the conditions of the assay, using endogenous kinases and ATP, the latter being added as a required substrate for the carboxylase reaction. The observation that [acetyl-CoA carboxylase]phosphatase 2 did not activate brain enzyme suggests that either the phosphatase is not active on brain enzyme or that the brain enzyme preparation was fully active, as isolated, and therefore cannot be further activated.

The enzyme appeared to be similar to the 265 kDa isozyme of acetyl-CoA carboxylase in its subunit molecular weight and immunological cross-reactivity, and distinct from the 280 kDa isozyme of heart (7). The minor protein band, with electrophoretic mobility slightly greater than that of the 265 kDa species, observed in preparations of chicken brain, appears to be immunologically related to the 265 kDa species (see Figs. 2 and 5) and may be proteolytically related. This is supported by the observation that acetyl-CoA carboxylase is very susceptible to proteolysis and brief treatment with trypsin cleaves a 5 kDa peptide (21).

Interestingly, the present study did not detect the presence of the 280 kDa isozyme in brain, a tissue that is not known to oxidize fatty acids. In other tissues, such as the adipose and lactating mammary gland that are predominantly lipogenic and non-ketogenic, the 280 kDa isozyme is not significant (8). Based on these observations, it is proposed that the 265 kDa isozyme of acetyl-CoA carboxylase is specific to lipogenic tissues and the 280 kDa is specific to ketogenic tissues. Thus brain, a tissue that is lipogenic and non-ketogenic, contains exclusively the 265 kDa species. In tissues such as the liver and brown adipose that are both lipogenic and ketogenic, both isozymic forms are seen (see Fig. 2) (8). In heart, a tissue that is highly ketogenic and non-lipogenic, it is the 280 kDa isozyme that is predominant (7).

Apparently the role of the 265 kDa isozyme of acetyl-CoA carboxylase is in providing malonyl-CoA for fatty acid synthesis. A role for the 280 kDa isozyme is not known yet. It is not likely that this isozyme is primarily involved in fatty acid synthesis since this is found predominantly in tissues that are ketogenic and non-lipogenic. Malonyl-CoA is present is significant quantities in tissues that are ketogenic and non-lipogenic (23). It is not known whether malonyl-CoA has any alternate metabolic roles in ketogenic tissues. In the absence of such, its role maybe regulatory. In this context it should be noted that malonyl-CoA is a potent inhibitor of mitochondrial carnitine palmitoyltransferase I, the rate-limiting enzyme in fatty acid oxidation (23). Thus the malonyl-CoA synthesized by the 280 kDa isozyme of acetyl-CoA carboxylase may be targeted toward regulation of ketogenesis via inhibition of carnitine palmitoyltransferase I. This would imply that, in tissues such as the liver and brown adipose that are both lipogenic and ketogenic, the two acetyl-CoA carboxylase isozymes may be compartmentalized.

The present study not only gave evidence for de novo synthesis of fatty acids in developing brain, but also suggested that, in lipogenic tissues that are not active in fatty acid oxidation, it is the 265 kDa, and not the 280 kDa, isozyme of acetyl-CoA carboxylase that is predominant.

We are grateful to Professors Salih J. Wakil and Robert A. Harris for valuable advice. The authors wish to thank Professor Roger C. Hoversland for reading this manuscript. This work was supported by a grant-in-aid from the American Heart Association (Indiana Affiliate).

Manuscript received 22 April 1991 and in revised form 9 July 1991.

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