Cloning of rat brain succinyl-CoA:3-oxoacid CoA-transferase cDNA

Regulation of the mRNA in different rat tissues and during brain development

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3-Oxoacid CoA-transferase, which catalyses the first committed step in the oxidation of ketone bodies, is uniquely regulated in developing rat brain. Changes in 3-oxoacid CoA-transferase activity in rat brain during the postnatal period are due to changes in the relative rate of synthesis of the enzyme. To study the regulation of this enzyme, we identified, with a specific polyclonal rabbit anti-(rat 3-oxoacid CoA-transferase), two positive cDNA clones (approx. 800 bp) in a λ gt11 expression library, constructed from poly(A)⁺ RNA from brains of 12-day-old rats. One of these clones (λ CoA3) was subcloned into M13mp18 and subjected to further characterization. Labelled single-stranded probes prepared by primer extension of the M13mp18 recombinant hybridized to a 3.6 kb mRNA. Rat brain mRNA enriched by polysome immunoadsorption for a single protein of size 60 kDa which corresponds to the precursor form of 3-oxoacid CoA-transferase was also found to be similarly enriched for the hybridizable 3.6 kb mRNA complementary to λ CoA3. Affinity-selected antibody to the λ CoA3 fusion protein inhibited 3-oxoacid CoA-transferase activity present in rat brain mitochondrial extracts. The 3.6 kb mRNA for 3-oxoacid CoA-transferase was present in relative abundance in rat kidney and heart, to a lesser extent in suckling brain and mammary gland and negligible in the liver. The specific mRNA was also found to be 3-fold more abundant in the brain from 12-day-old rats as compared with 18-day-old fetuses and adult rats, corresponding to the enzyme activity and relative rate of synthesis profile during development. These data suggest that 3-oxoacid CoA-transferase enzyme activity is regulated at a pretranslational level.

INTRODUCTION

Ketone bodies, acetoacetate and D-3-hydroxybutyrate, are utilized by extrahepatic tissues as respiratory fuels and precursors of fatty acid synthesis (for reviews see [1,2]). During starvation and diabetes, synthesis of ketone bodies by the liver is accelerated to meet the energy requirements of the body, especially the brain [3]. The oxidation of ketone bodies occurs in the mitochondria and is catalysed by the sequential action of D-3-hydroxybutyrate dehydrogenase, succinyl-CoA:3-oxoacid CoA-transferase (3-oxoacid CoA-transferase, EC 2.8.3.5) and acetoacetyl-CoA thiolase. The key enzyme in this oxidative pathway is 3-oxoacid CoA-transferase, which catalyses the first committed step converting acetoacetate to acetoacetyl-CoA in the presence of succinyl-CoA. In the liver the enzyme is not present, sparing ketone bodies to be utilized by peripheral organs.

Ketone bodies also play an important role in cerebral metabolism of developing rats, since they constitute the major oxidative substrate during the suckling period. This is consistent with the unique developmental pattern of the ketone-body-metabolizing enzymes. The activity of these enzymes is relatively high at birth, increases about 3–5-fold and then declines at weaning to the adult level [4,5]. This is in contrast with the development of other mitochondrial enzymes, whose activities increase

gradually from a low level at birth to the adult level during the first 3 weeks. Recently, we have demonstrated that the changes in the activity of 3-oxoacid CoA-transferase are due to changes in the relative rate of synthesis of this enzyme [6]. However, the molecular mechanism responsible for these changes in the developing rat brain and the differential expression of this enzyme in different tissues is unknown. To address these questions, we isolated a cDNA clone for 3-oxoacid CoA-transferase and used this to estimate the level of mRNA in different tissues and at different stages of rat brain development.

EXPERIMENTAL

Construction and screening of a recombinant cDNA expression library

RNA was isolated from brains of 12-day-old rats by the guanidine thiocyanate procedure [7]. Poly(A)⁺ RNA was obtained by column chromatography on oligo(dT)-cellulose [8]. A recombinant cDNA library in the expression vector λ gt11 was constructed from brain poly(A)⁺ RNA of 12-day-old rats and screened essentially as described by Huynh *et al.* [9]. Approx. 1×10^6 plaque-forming units were screened initially. The plaques were treated with isopropyl β -D-thiogalactopyranoside to induce expression from the *lac* promoter of the vector

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and transferred to nitrocellulose membranes. The recombinant plaques were screened with a 1:1500 dilution of a specific polyclonal antibody to rat brain 3-oxoacid CoA-transferase [4] from which antigenic determinants to *E. coli* and coliphage λ proteins were removed on an affinity column of *E. coli* lysate from BNN 97 linked to CNBr-treated Sepharose 4B [9]. Positive plaques containing 3-oxoacid CoA-transferase determinants were identified with peroxidase-conjugated goat anti-(rabbit IgG) [10]. Two positive clones (λ CoA3 and λ CoA4) identified at the initial round of screening were subjected to three additional rounds of screening until all plaques gave a positive signal.

Isolation of antibodies specific for the λ CoA3 fusion protein

Nitrocellulose filters containing proteins produced by λ gt11 (control) or λ CoA3 recombinant phage were used to affinity-select antibodies from anti-(3-oxoacid CoA-transferase) serum [11]. Affinity-selected antibodies were concentrated and reacted with 3-oxoacid CoA-transferase activity (20 munit/ml) present in a crude rat brain mitochondrial extract [6,12].

Isolation of cDNA insert and subcloning into M13mp18

Bacteriophage λ gt11 recombinant DNA from one of the immunoreactive clones (λ CoA3) was prepared by the plate lysis procedure [13]. The DNA was then digested with *Eco*RI and the insert, purified by electroelution from a 1% agarose gel, was inserted into the unique *Eco*RI site of M13mp18 [14]. Recombinant clones grown on *E. coli* strain JM 105 were recognized as colourless plaques. Ten representative plaques were further purified and the orientation of the inserts relative to each other was determined. Single-stranded virion DNAs containing cDNA inserts in both orientations were isolated and used for preparing labelled single-stranded DNA probes by primer extension.

Enrichment of CoA-transferase mRNA by polysome immunoadsorption

Polysomes were prepared from 100 brains of 10-day-old rats according to the procedure of Kraus & Rosenberg [15]. Trichodermin (1 μ g/ml) was substituted for cycloheximide [16] and heparin was used at a concentration of 2 mg/ml to reduce nonspecific binding and entrapment of unwanted polysomes [17]. The polysomes were reacted with rabbit anti-(rat 3-oxoacid CoA-transferase) at 4 °C for 2–3 h and the antigen–antibody complex was isolated on a Protein A–Sepharose CL-4B column. Enriched mRNA for 3-oxoacid CoA-transferase was obtained by dissociation of the complex with 25 mM-Tris/HCl (pH 7.5)/20 mM-EDTA. Poly(A)⁺ RNA was isolated on oligo(dT)–cellulose [8].

Hybridization of cDNA to RNA

RNA was resolved on a 0.8% agarose gel containing 2.2 M-formaldehyde [18]. The gel was transferred to nitrocellulose membrane in 20 $\times$ SSC (3 M-NaCl/0.3 M-sodium citrate) and hybridized to the single-stranded probes (2 $\times$ 10⁶ d.p.m./ml) of the cDNA insert in M13mp18. Hybridization conditions were similar to those described by Hod *et al.* [19].

Translation *in vitro* of poly(A)⁺ RNA

Poly(A)⁺ RNA was translated *in vitro* using the rabbit reticulocyte lysate system and L-[³⁵S]methionine (New England Nuclear) according to the manufacturer's protocol. The total volume of the reaction mixture was 25 μ l for samples containing less than 3 μ g of poly(A)⁺ RNA and 50 μ l for samples containing 3–6 μ g of poly(A)⁺ RNA. The reaction mixture was incubated at 30 °C for 60 min and two 1 μ l aliquots were removed for determining the pattern of total translational products on SDS/polyacrylamide gels and for radioactivity incorporated into total proteins by trichloroacetic acid precipitation. The remaining volume was reacted with 10 μ l of antiserum at 37 °C for 60 min and at 4 °C overnight. The antigen–antibody complex was isolated on Protein A–Sepharose CL 4B and subjected to SDS/polyacrylamide-gel electrophoresis.

RESULTS AND DISCUSSION

Changes in 3-oxoacid CoA-transferase activity in the developing rat brain are due to changes in the relative rate of synthesis of the enzyme and not due to post-translational regulation of its catalytic efficiency. However, the mechanisms responsible for regulating the expression of 3-oxoacid CoA-transferase in different tissues and at different stages of rat brain development are not known. As a first step toward investigating the molecular mechanisms responsible for modulating enzyme activity, in this study we have isolated a cDNA clone for 3-oxoacid CoA-transferase and used it to determine the level of the mRNA in different tissues and at different stages of rat brain development.

An expression library in the bacteriophage vector λ gt11 was constructed from brain poly(A)⁺ RNA from 12-day-old rats and screened with a polyclonal antibody to the rat brain enzyme. Positive signals were detected with peroxidase-conjugated goat anti-(rabbit IgG). Brain poly(A)⁺ RNA from 12-day-old rats was used to construct the library, since the relative rate of synthesis (0.05%) is maximal between postnatal days 2 and 12 [6]. Initial screening was carried out at a high density to increase the probability of detecting the low abundance 3-oxoacid CoA-transferase cDNA. Approx. 1 $\times$ 10⁶ plaque-forming units were screened. The antiserum used for screening the library was specific for 3-oxoacid CoA-transferase [20], and was capable of detecting as little as 0.1 ng of the protein at a 5000-fold dilution. For screening the library, the antiserum was diluted 1500-fold to reduce non-specific detection of clones coding for proteins other than 3-oxoacid CoA-transferase. On the first round of screening two clones were identified (λ CoA3 and λ CoA4). One of these (λ CoA3) consistently produced a more intense reaction. Both clones were subjected to three additional rounds of screening until all plaques were positive. Plaques for each additional round of screening were plated at lower densities compared with the previous ones. At the last round of screening duplicate filters of plaque-purified λ CoA3 were probed with antiserum and preimmune serum. Positive signals were obtained with the antiserum but not with preimmune serum (results not shown).

The size of the cDNA insert was determined after digesting the recombinant DNA with *Eco*RI. Both contained inserts of approx. 800 bp; therefore the clone

producing the more intense signal (λ CoA3) was used for further characterization. The insert was subcloned into the single-stranded bacteriophage vector M13mp18 after its separation from the vector on a 1% agarose gel. Single-stranded virions containing cDNA insert in both orientations were isolated and used to prepare labelled single-stranded probes for hybridization analysis.

3-Oxoacid CoA-transferase is a mitochondrial protein composed of two identical subunits. The subunit molecular mass of the rat brain enzyme is approx. 55 kDa [21]. Since most mitochondrial proteins are synthesized as precursor polypeptides containing an *N*-terminal extension (signal peptide) of approx. 2–10 kDa in size [22,23], the molecular size of newly synthesized 3-oxoacid CoA-transferase should be larger than that of the mature protein. Cell-free translation of rat brain poly(A)⁺ RNA revealed that a single polypeptide migrating at a molecular mass of about 60 kDa on SDS/polyacrylamide gels was immunoprecipitated by anti-(3-oxoacid CoA-transferase) serum (Fig. 1, lanes 6 and 7). Thus, newly synthesized 3-oxoacid CoA-transferase contains a signal peptide that is about 5 kDa in size. The molecular mass of the precursor enzyme from rat kidney and heart was similar to that of rat brain. In the liver, which does not contain 3-oxoacid CoA-transferase activity, the precursor form of 3-oxoacid CoA-transferase (60 kDa) was not synthesized in translation assays *in vitro* (results not shown).

To demonstrate that λ CoA3 contains sequences

Fig. 1. Translation *in vitro* of total and immunoenriched poly(A)⁺ RNA from 10-day-old rats

Total and polysome immunoenriched poly(A)⁺ RNA prepared from brains of 10-day-old rats as described under 'Experimental' were translated in a rabbit reticulocyte lysate system, immunoprecipitated with anti-(3-oxoacid CoA-transferase) serum and electrophoresed on a 9% running, 6% stacking SDS/polyacrylamide gel. Translation was carried out in the absence of added poly(A)⁺ RNA (lanes 1 and 4), or presence of poly(A)⁺ RNA [0.9 μ g total brain (lane 5), 5.0 μ g total brain (lanes 2 and 6) and 0.9 μ g polysome immunoenriched (lanes 3 and 7)]. Lanes 1–3 correspond to a 1 μ l aliquot of the L-[³⁵S]-methionine-labelled total translation products and lanes 4–7 correspond to the immunoprecipitated L-[³⁵S]-methionine-labelled precursor form of 3-oxoacid CoA-transferase (60 kDa). The numbers refer to the migration of the molecular mass markers; Tr. refers to the migration of purified rat brain 3-oxoacid CoA-transferase (55 kDa).

complementary to 3-oxoacid CoA-transferase mRNA, two different lines of evidence were employed. We first compared total brain poly(A)⁺ RNA and poly(A)⁺ RNA specifically enriched for 3-oxoacid CoA-transferase by cell-free translation and Northern blot hybridization. Evaluation of L-[³⁵S]methionine-labelled *in vitro* translation products of total brain poly(A)⁺ RNA and polysome immunoenriched poly(A)⁺ RNA for 3-oxoacid CoA-transferase on SDS/polyacrylamide gels indicated that a single protein band migrating at a molecular size of 60 kDa was substantially increased for the enriched poly(A)⁺ RNA compared with total brain poly(A)⁺ RNA (Fig. 1, lanes 3 and 2, respectively). This band was identified to be the precursor form of 3-oxoacid CoA-transferase since it was immunoprecipitated by the specific antiserum (Fig. 1, lane 7). The amount of the immunoprecipitated precursor obtained after translation of only 0.9 μ g of immunoenriched mRNA was significantly greater than that obtained after translation of 5 μ g of total brain poly(A)⁺ RNA. If λ CoA3 indeed coded for 3-oxoacid CoA-transferase protein, a similar increase in the mRNA transcript hybridizing to the ³²P-labelled λ CoA3 probe for the enriched mRNA should also be observed. Additionally, the size of the mRNA transcript hybridizing to λ CoA3 should be at least 1.8–2.0 kb, which is sufficient to code for a protein of molecular mass of about 60 kDa. Northern blot analysis of total brain and enriched mRNA revealed that a 3.6 kb mRNA hybridized to the labelled single stranded cDNA in both cases. This size is large enough to code for precursor 3-oxoacid CoA-transferase. The amount of hybridizable mRNA in 0.5 μ g of enriched mRNA preparation was significantly greater than that in 10 μ g of total brain poly(A)⁺ RNA (Fig. 2), which is similar to the *in vitro*

Fig. 2. Northern blot analysis of total immunoenriched poly(A)⁺ RNA from 10-day-old rats

Poly(A)⁺ RNA [0.5 μ g total brain (lane 1), 0.5 μ g immunoenriched brain (lane 2) and 10 μ g total brain (lane 3)] was denatured in formaldehyde and subjected to Northern blot analysis as described under 'Experimental'. The numbers refer to molecular mass markers (kb).

translation results. This indicated that precursor 3-oxoacid CoA-transferase (60 kDa) is encoded by the mRNA complementary to λ CoA3. We also determined the ability of affinity-selected antibody to the fusion protein produced by recombinant λ CoA3 to inhibit 3-oxoacid CoA-transferase enzyme activity present in rat brain mitochondrial extracts. This affinity-selected antibody specifically inhibited 3-oxoacid CoA-transferase activity, whereas similar concentrations of affinity-selected antibody by proteins produced by control λ gt11 phage had no inhibitory effect on enzyme activity (Table 1). These data together provide strong support for the authenticity of the λ CoA3 cDNA clone.

As a first step toward determining the mechanisms by which enzyme activity in different tissues and during different stages of rat brain development is regulated, Northern blot analysis of RNA isolated from different rat tissues and from the rat brain at different stages of development was carried out with the λ CoA3 cDNA clone. The different tissues that were analysed, including liver, suckling brain, mammary gland, kidney and heart, represent a range of activity of 3-oxoacid CoA-transferase, from negligible in the liver to maximal in the kidney and heart. Northern blot analysis of RNA from these tissues indicated that 32 P-labelled λ CoA3 cDNA hybridized to a 3.6 kb RNA from suckling brain, adult kidney, mammary gland and adult heart (Fig. 3, lanes 2–5). No signal was observed with RNA from the liver (Fig. 3, lane 1), which contains very low levels of 3-oxoacid CoA-transferase activity. Comparison of the intensity of the hybridizable mRNA in lanes containing identical amount of poly(A)⁺ RNA from suckling brain, adult kidney and mammary gland (Fig. 3, lanes 2–4) and in the lane containing significantly less poly(A)⁺ RNA from the heart (Fig. 3, lane 5), indicates that 3-oxoacid CoA-transferase mRNA is expressed at high levels in the kidney and heart. The suckling brain and mammary gland contain lower amounts of 3-oxoacid CoA-transferase mRNA. The enzyme activity is also highest in the

adult kidney and heart and about 3–4-fold higher than in suckling brain and mammary gland [1,24]. This suggests that the expression of 3-oxoacid CoA-transferase is

Fig. 3. Tissue distribution of 3-oxoacid CoA-transferase mRNA

Northern blot analysis of poly(A)⁺ RNA from 20 μ g of adult rat liver (lane 1), 10 μ g of brain from 10-day-old rats (lane 2), 10 μ g of adult rat kidney (lane 3), 10 μ g of rat mammary gland (lane 4) and total RNA from 50 μ g of adult rat heart (lane 5) was carried out as described in Fig. 2.

Table 1. Inhibition of 3-oxoacid CoA-transferase activity in rat brain mitochondrial extract by affinity-selected antibody isolated by the fusion protein produced by the λ CoA3 recombinant

Affinity-selected antibody was added to 3-oxoacid CoA-transferase (20 munits/ml) in a total volume of 200 μ l, and incubated at 37 °C for 45 min followed by incubation at 4 °C for 5 h. The antigen–antibody complex was immunoprecipitated with Protein A–Sepharose CL-4B, and the activity present in the supernatant was determined.

Volume of affinity-selected antibody (μ l)	3-Oxoacid CoA-transferase activity present in supernatant (%)
λ CoA3	
0	100
25	33
50	0
λ gt11 (control)	
0	100
25	112
50	91

Fig. 4. Developmental regulation of 3-oxoacid CoA-transferase mRNA in the brain

Total RNA (50 μ g) from 18-day foetal (lane 1), 10-day-old (lane 2) and adult (lane 3) rat brain was subjected to Northern blot analysis as described in Fig. 2.

regulated at a pretranslational step. Northern blot analysis of total RNA from brains of 18-day foetal, 10-day-old and adult rats (Fig. 4) indicated that the amount of 3-oxoacid CoA-transferase mRNA in 10-day-old rats was approximately 3-fold higher than in foetal or adult rats. This is consistent with the changes observed in the enzyme activity and the relative rate of synthesis during the developmental period, again suggesting regulation at a pretranslational step.

In conclusion, we have isolated a 800 bp cDNA clone for 3-oxoacid CoA-transferase. This clone hybridizes with a 3.6 kb RNA from the rat mammary gland, brain, kidney and heart, but not from the liver. The amount of 3-oxoacid CoA-transferase mRNA from brains of 10-day-old rats is 3-fold higher than of foetal and adult rats, consistent with the enzyme activity and relative rate of synthesis data. These data suggest that a pretranslational step regulates the enzyme activity of 3-oxoacid CoA-transferase. Isolation and characterization of the gene and studies of the various regulatory steps between transcription of the gene and translation of the mRNA would provide a better understanding about the regulatory mechanism controlling 3-oxoacid CoA-transferase enzyme activity.

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