Rat ATP Citrate-Lyase

MOLECULAR CLONING AND SEQUENCE ANALYSIS OF A FULL-LENGTH cDNA AND mRNA ABUNDANCE AS A FUNCTION OF DIET, ORGAN, AND AGE*

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ATP citrate-lyase is the primary enzyme responsible for the synthesis of cytosolic acetyl-CoA in many tissues. We have isolated a full-length cDNA copy of 4.3 kilobase pairs encoding the ATP-citrate-lyase mRNA by screening rat liver cDNA library using oligonucleotide probes designed from peptide sequences obtained from the purified rat enzyme. Expression of this cDNA in bacteria, followed by immunoblotting with antibody directed against the ATP citrate-lyase, further demonstrated the identity of this clone. Nucleic acid sequence data indicate that the cDNA contains the complete coding region for the enzyme, which is 1100 amino acids in length with a calculated molecular weight of 121,293. RNA blot analysis indicated an mRNA species of about 4.3 kilobase pairs in livers of Chow-fed rats. Rats maintained on low fat, high carbohydrate diets exhibited a striking increase (50-fold) in the level of liver ATP citrate-lyase mRNA as compared with the control animals maintained on a normal diet. The tissue distribution of this mRNA in Chow-fed animals revealed a relatively high abundance of the message in liver and adrenal, moderate levels were found in lung, brain, and large intestine with only trace amounts of the message in small intestine, stomach, testis, spleen, pancreas, kidney, and heart. During rat development, the ATP citrate-lyase mRNA was relatively high in the liver at parturition, followed by a reduction in its level during suckling. Higher amounts of the mRNA were detected again in adult animals. The isolation and characterization of the mRNA for ATP citrate-lyase will allow further studies on the reaction mechanism and metabolic regulation of this key enzyme in lipogenesis and cholesterogenesis.

ATP citrate-lyase is a cytoplasmic enzyme widely distributed in mammalian tissues. The enzyme is a tetramer (molecular weight about 440,000) of four apparently identical subunits (1). It catalyzes the formation of acetyl-CoA and oxaloacetate from citrate and CoA with a concomitant hydrolysis of ATP to ADP and phosphate. The product, acetyl-CoA, serves several important biosynthetic pathways, including lipogenesis and cholesterogenesis. Supporting evidence for its central role in de novo lipid synthesis is supplied by studies with hydroxycitrate, a specific inhibitor of ATP citrate-lyase. This compound reduces fatty acid and cholesterol synthesis in a variety of tissues including liver and adipose tissue (2). In nervous tissue ATP citrate-lyase may be involved in the biosynthesis of acetylcholine (3). The mechanism of the ATP citrate-lyase reaction involves the phosphorylation by Mg-ATP of a histidine residue in the active site (4–6). In addition to the catalytic site, serine residues can be phosphorylated at two chemically distinct "regulatory" sites located on two tryptic peptides (7). Phosphorylation of these sites has been observed in vitro in response to both cAMP dependent (A-site) (8) and independent protein kinases (9) (B-site) as well as in vivo, in response to insulin and glucagon (10, 11). The physiological function of this regulatory phosphorylation is unclear. The ability to phosphorylate at either one of the regulatory sites depend on the phosphorylation state of the other regulatory site (7). Although phosphorylation at the A-site decreases enzyme activity under certain conditions (12), major regulation of ATP citrate-lyase activity is probably not by phosphorylation/dephosphorylation but by altering the amount of enzyme. Enzyme levels in liver and adipose tissue vary over a wide range under the control of nutrients and hormones (13), declining sharply in starved animals and rising many fold when the animals are fed a high carbohydrate/low fat diet (14). These changes in ATP citrate-lyase activity are due to alterations in the rate of ATP citrate-lyase biosynthesis (14), but the level of its regulation is largely unknown (15). Since the ATP citrate lyase is a potential target for hypolipidemic intervention, we have isolated and sequenced a cDNA clone of the rat liver enzyme. The present study also reports the abundance of the mRNA in tissues of adult rats and animals during development. Furthermore, the sequence information allowed us a comparison with sequences of other enzymes catalyzing related reactions or having similar regulatory elements. Based on these comparisons, we speculate on substrate binding sites and regulatory elements in the rat ATP citrate-lyase.

EXPERIMENTAL PROCEDURES

Purification of ATP-citrate Lyase—Male rats (950–100 g) were fasted for 24 h and then refed a fat-free high carbohydrate diet for 24 h. Livers were homogenized in 250 mM sucrose, 10 mM β-mercaptoethanol, 50 mM Tris HCl, pH 7.4 (1:4, w/v). The homogenate was centrifuged at 15,000 × g for 20 min to remove cell debris and cell organelles. The supernatant was collected and centrifuged at 105,000
ATP citrate-lyase was precipitated from the supernatant solution with (NH₄)₂SO₄ at 0-40% of saturation. The precipitate was collected by centrifugation and resuspended in a minimal volume of 50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 10 mM β-mercaptoethanol, and dialyzed overnight against the same buffer. The resolubilized fraction was then loaded onto a C26 Pharmacia LKB Biotechnology Inc. fast-flow DEAE column, and eluted with a 0-400 mM NaCl gradient at a flow rate of 2 ml/min over 200 ml. Enzyme recovery was determined by the molydate color-dense coupled assay (16) and active fractions pooled. Following dialysis against 50 mM phosphate buffer, pH 7.0, 2 mM MgCl₂, 10 mM β-mercaptoethanol, the eluate was passed down a Procion yellow MX6G-Sepharose CL4B column. ATP citrate-lyase was bound to the column and eluted quantitatively. The column was washed with buffer containing 1 mM ATP to remove a variety of contaminating proteins and then 1 mM ATP, 20 mM tripotassium citrate, pH 7.0, was used to specifically elute ATP citrate-lyase. The enzyme eluted as a single band, with molecular weight of 120,000 on SDS-polyacrylamide gel electrophoresis. The specific activity was 8 units/mg (1 unit of enzyme produces 1 µmol of NAD⁺/min at 25 °C in assay buffer containing 10 mM β-mercaptoethanol at 25 °C, pH 8).

Purification of Peptides and Protein Sequencing—Prior to sequence analysis both intact proteins and peptide fragments were isolated by reversed-phase-HPLC (RP-HPLC) using a 2 x 30-mm aquapore RP-300 cartridge (Applied Biosystems). Solvent A was 0.06% trifluoroacetic acid in water, solvent B 0.06% trifluoroacetic acid in 50% solvent A. Gradients of 0-70% solvent B were run in 35 min at 0.2 ml/min. Absorbance was recorded at 215 nm. Amino acid sequencing was carried out using the Applied Biosystems 477A protein sequencer. Native protein (250 µg) was de-salted by RP-HPLC and 20 µg were applied to the sequencer (for N-terminal determination). De-salted protein fractions from both native and S-carboxymethylated ATP citrate-lyase were reduced in volume and 100 µg of each were cleaved with cyanogen bromide (100 µg in 50 µl of 70% trifluoroacetic acid for 15 h). The resulting peptides were separated by RP-HPLC and their amino acid sequences determined.

Preparation of Oligonucleotide Probes—The peptide sequences were used to design oligonucleotide probes according to the preferred codon utilization for mammalian genes. Probes of 38-42 nucleotides in length were synthesized using an Applied Biosystems 380B DNA synthesizer. The oligonucleotides were purified by electrophoresis through 20% polyacrylamide gels containing 7 M urea. The purified oligonucleotides were then 5'-end-labeled using [γ-32P]ATP and T4 polynucleotide kinase (New England BioLabs).

Preparation and Screening of the cDNA Library—The cDNA library was constructed from total cellular RNA. RNA was extracted from rat liver by the method of Chirgwin et al. (22) and analyzed on agarose gel electrophoresis. The resulting RNA samples were denatured, applied to nitrocellulose filters, and hybridized to 32P-labeled cDNA (18). Each filter contained a liver RNA sample that was diluted over a 100-fold concentration range as a reference standard. Autoradiograms of the slots show the position of RNA standard markers. The results presented are representative of at least three independent hybridization experiments.

RESULTS AND DISCUSSION

Deduced Amino Acid Sequences of ATP Citrate-Lyase—We initially screened 100,000 recombinants of rat liver cDNA library with a 42-mer oligonucleotide probe (base pairs 1821-1860) designed from the sequence of a cyanogen bromide peptide fragment. This screening yielded five clones which hybridized with oligonucleotide probes derived from the sequences of two other peptide fragments (base pairs 1530-1566 and 2595-2638) obtained from different regions of the enzyme. A preliminary analysis by agarose gel electrophoresis indicated the length of the cDNA inserts was about 4.3 kilobase pairs. These clones were then used to probe rat liver mRNA by RNA Northern blot analysis and were found to hybridize to an mRNA of approximately 4.3 kilobase pairs (Fig. 1A). Thus, it appeared that the recombinant cDNAs were essentially full-length copies of the corresponding RNA. The identity of the recombinant cDNA was established by expression studies. The cDNA clones were transfected into E. coli AR58 cells and the cells induced to express the ATP citrate-lyase protein. Western blot analysis with a rabbit polyclonal antisera prepared against the rat ATP citrate-lyase demonstrated a M, 120,000 protein (Fig. 1B), supporting the identity of the recombinant cDNA. The probe did not cross-hybridize detectably with yeast RNA.

Animals. Male Sprague-Dawley rats (120-140 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and allowed free access to Purina Rat Chow.

FIG. 1. A, ATP citrate-lyase mRNA in rat liver by RNA Northern blot hybridization. Total liver RNA was extracted from chow-fed control rats or rats fasted for 1 day and then refed high carbohydrate, low fat Purina diet for 2 days. Animals were sacrificed at 9:00 A.M. Samples containing 5 µg of total cellular RNA were denatured with glyoxal, electrophoresed in 1.1% agarose gels, blotted to nitrocellulose, and probed with either E. coli AR58 or E. coli AR581, carrying the c1 ssi TS mutation. The cells were grown at 32 °C (plasmids were transfected into E. coli AR58 cells and the cells induced to express the ATP citrate-lyase protein by rapidly increasing the temperature to 42 °C. The cell extract was examined on Western blot analysis using rabbit antibody against native rat ATP citrate-lyase and compared with either E. coli extract with vector alone or purified rat ATP citrate-lyase protein.
evidence that this recombinant clone represents rat ATP citrate-lyase cDNA.

**Primary Structure of Rat ATP Citrate-Lyase**—A restriction map and sequencing strategy for the cDNA clone are shown in Fig. 2A. The complete nucleotide sequences of both strands were determined. There was only one long open reading frame, beginning at a methionine codon (nucleotide 1) and ending at the stop codon TGA (nucleotide 3,300) (Fig. 2B). The cDNA insert also contained 5' and 3' nontranslated regions of 72 and 926 nucleotides, respectively. The latter region contains the polyadenylation signal, AATAAA, beginning at nucleotide 4205. The deduced amino acid sequence contains 1,100 residues with a calculated Mr of 121,293. This value is in good agreement with the Mr 120,000-130,000 estimated for rat ATP citrate-lyase by gel electrophoresis. We confirmed our reading frame by additional peptide sequencing. Eleven peptides were isolated from cyanogen bromide digests of rat liver ATP citrate-lyase by HPLC. The amino-terminal amino acid sequences were determined for each peptide (Table I), and compared to the amino acid sequence deduced from our overlapping cDNA clones. In all cases there was a perfect match (Fig. 2B).

The amino terminus of the protein was blocked. To confirm that our cDNA clones contained the correct amino terminus, a Styl/Acl fragment of 627 base pairs corresponding to the first 209 amino acids of the deduced sequence was inserted into pMG3 vector2 and transferred to E. coli AR56 cells which were then induced to express the protein. The cell extract was assayed for the production of a protein reactive with rabbit antibody (Fig. 2B), and the amino-terminal amino acid sequence was determined for each clone (Table I). Eleven peptides were isolated from cyanogen bromide digests of rat liver ATP citrate-lyase by HPLC. The amino-terminal amino acid sequences were determined for each peptide (Table I), and compared to the amino acid sequence deduced from our overlapping cDNA clones. In all cases there was a perfect match (Fig. 2B).

Analysis of the ATP citrate-lyase sequence by the method of Kyte and Doolittle (24) revealed hydrophilic regions at the amino and carboxyl termini of the molecule comprising approximately 150 amino acids at each end. The rest of the molecule contains a mixture of hydrophobic and hydrophilic amino acids. Secondary structure predictions (Chou and Fasman, (25)) indicate that the ATP citrate-lyase is 56% $\beta$-sheet and 44% $\alpha$-helical structure. ATP citrate-lyase is phosphorylated in vivo in response to insulin, glucagon, $\beta$-adrenergic agonists, and transforming growth factor-$\beta$ (10, 11) and in vitro by CAMP-dependent protein kinase (6). This latter phosphorylation site is the first serine in the peptide TASFS-ESR (amino acids 452-459) (26, 27), with serine 454 being the phosphorylation target. The consensus CAMP-dependent protein kinase site contains a hydrophobic residue carboxyl-terminal to the site and 2 basic residues on the amino-terminal side (28).

One of the most striking findings is the strong similarity of the deduced ATP citrate-lyase amino acid sequence with the $\alpha$ chain of succinyl-CoA synthetase of E. coli (30). Analysis using the FASTA program (31) indicates that the ATP citrate-lyase (residues 560–800) and bacterial succinyl-CoA synthetase $\alpha$ chain (residues 60–290) shows a statistically significant sequence identity of 39% (Fig. 3). The inclusion of conservative replacements increases the degree of similarity to 69%. The two enzymes catalyze similar reactions. Succinyl-CoA synthase involves (i) autophosphorylation of the enzyme by ATP, (ii) phosphorylation of succinate by phosphoenzyme, and (iii) attack of CoA on succinate phosphate to form succinyl-CoA. With ATP citrate-lyase the pattern is identical as far as the formation of citryl-CoA. However, this unstable compound undergoes a retro-Claisen reaction to produce acetyl-CoA and oxaloacetate.

Detailed comparison of the two sequences reveals a five amino acid identity, GHAGA (labeled ATP “A” in Fig. 3), which surrounds the catalytic histidine residue in succinyl-CoA synthetase. This histidine is autophosphorylated by ATP. Since the ATP citrate-lyase reaction also involves a phosphohistidine intermediate, it seems reasonable to suggest that histidine 760 is the site of catalytic phosphorylation in ATP citrate-lyase. Further comparisons revealed that within the regions of similarity between the two enzymes there is a sequence, (labeled ATP “B” in Fig. 3) which is similar to the consensus sequence for part of the ATP binding pocket (32). The B sequence in succinyl-CoA synthetase and adenylate kinase is characterized by a K/K-X$_{-3}$G-X$_{3}$ (hydrophobic), consensus sequence followed by a negatively charged residue (Glu-210) that is implicated in Mg$^{2+}$ binding. The hydrophobic residues (amino acids 205–208) form a $\beta$ sheet making contacts with the one face of the adenine ring (32). The region (amino acids 700–720) in ATP citrate lyase is predicted to have similar structure and could therefore form part of the ATP binding site. A model for a consensus CoA binding site has been proposed based on the available x-ray crystallographic data from citrate synthase and homologues between citrate synthase, acetyl-CoA carboxylase, and propionyl-CoA carboxylase (33, 34).

The consensus sequence for the adenine binding loop and $\beta$-diphosphate contacts is V/L-X-G-X-G-X-V-X/R/K. One section of the ATP citrate-lyase sequence, marked in Fig. 3, conforms to this pattern. It should be stressed, however, that we have no direct evidence that this is the recognition loop.

**mRNA Abundance as a Function of Diet, Organ, and Age**—The relative amount of mRNA for ATP citrate-lyase in 12 different adult male rat tissues was determined for animals maintained overnight on a normal rat chow diet and sacrificed in the morning. The level of ATP citrate-lyase mRNA is the highest in liver (Fig. 4). The adrenals, lung, brain, and large intestine also are relatively rich in lyase mRNA containing 56, 31, 25, and 30% of that in the liver, respectively. The relative levels of ATP citrate-lyase mRNA in other tissues ranged from as little as 3% to as much as 16% of the levels in liver (Fig. 4). The ATP citrate-lyase mRNA level was also measured in livers of rats fed a high carbohydrate/low fat diet, following a 24-h fasting period (starved/refed animals). This regimen is known to strongly stimulate the activity of all lipogenic enzymes including ATP citrate-lyase due to a higher rate of enzyme synthesis (14). Fig. 1A shows that such a treatment results in a dramatic (50-fold) increase in hepatic ATP citrate-lyase mRNA abundance when compared with chow-fed animals. Similar increases were reported earlier in mice following an analogous dietary regimen (15).

ATP citrate-lyase mRNA abundance was also measured in liver and three extrahepatic tissues during development. Since the yield of RNA from individual tissues at early stages of development was relatively low, organs were pooled from animals of the same age. Thus, estimates of ATP citrate-lyase mRNA levels represent average values that mask possible differences between individual animals. Hepatic ATP citrate-lyase mRNA levels were relatively high at parturition, diminished rapidly during the suckling period (days 1–14) and were found high again after weaning in young adult animals (Fig.

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FIG. 2. A, strategy for nucleotide sequence analysis of rat ATP citrate-lyase cDNA. The top line indicates the nucleotide sequence that was determined from the cDNA insert that was cloned into the Bluescript vector. Restriction endonuclease sites for subcloning and sequence analysis are indicated by vertical lines. The solid lines below represent reactions primed with either the universal Bluescript primer or synthetic oligonucleotides complementary to portions of the ATP citrate-lyase cDNA sequence. B, nucleotide and deduced amino acid sequences of ATP citrate-lyase cDNA and protein sequence, respectively. Nucleotide residues are numbered in the 5' to 3' direction, beginning with the 1st residue of the ATG triplet encoding the initiation codon for methionine 1. The nucleotides to the 5' of residue 1 are indicated by negative numbers; the number of the nucleotide residue is given at the right end of each line. Deduced amino acid residues are indicated beginning with the initiation methionine. The regions identifying partial amino acid sequences of ATP citrate-lyase peptides are underlined and numbered sequentially. The translation termination codon is indicated by an asterisk. The location of one of the regulatory phosphorylation sites, identified earlier (8, 27), is indicated (P).
Adipose tissue are strongly affected by the composition of the diet, being high fat/low carbohydrate during suckling, and high again after weaning (35-39). Activities of hepatic ATP citrate-lyase follow this pattern (37, 38). Lipogenesis is also an important pathway in the developing brain, particularly with respect to myelination. In contrast to liver, lipogenesis in brain increases transiently during the 1st week of suckling reaching maximal rates 5-10 days after parturition (23, 43). It is of interest that transcriptional control and relations between its structure and function of ATP citrate-lyase will provide the tools to examine its transcription as described under "Experimental Procedures." Cloned [3P]-labeled ATP citrate-lyase cDNAs (38) were employed. Several different exposures of the autoradiograms of the hybridized filters were made and analyzed by quantitative scanning densitometry. (Average of three different replicates with less than 10% differences.)

### Table I

Amino acid sequences of peptides isolated from cyanogen bromide digests of rat ATP citrate-lyase

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cleavage position</th>
<th>Residues identified</th>
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<tr>
<td>CB1</td>
<td>Met-278</td>
<td>VAGGAGSVVYSDTI</td>
</tr>
<tr>
<td>CB2</td>
<td>Met-327</td>
<td>TKEKHDPD</td>
</tr>
<tr>
<td>CB3</td>
<td>Met-328</td>
<td>VVPTTGD</td>
</tr>
<tr>
<td>CB4</td>
<td>Met-559</td>
<td>KKHPEVDVLIN</td>
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<td>CB5</td>
<td>Met-580</td>
<td>NYAOIKIAI</td>
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<tr>
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<td>Met-641</td>
<td>LDNILASKLYRP</td>
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<tr>
<td>CB7</td>
<td>Met-665</td>
<td>SNEINHSSRTTDGYEGVAI</td>
</tr>
<tr>
<td>CB8</td>
<td>Met-856</td>
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<td>CB10</td>
<td>Met-1035</td>
<td>CRNGGSFTRREA</td>
</tr>
<tr>
<td>CB11</td>
<td>Met-1066</td>
<td>FIGHYLDQKR</td>
</tr>
</tbody>
</table>

5. Peptides are listed in amino-terminal to carboxyl-terminal order according to assignments within the cDNA-derived sequence. For experimental details see "Experimental Procedures."

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**REFERENCES**

Rat ATP citrate-lyase. Molecular cloning and sequence analysis of a full-length cDNA and mRNA abundance as a function of diet, organ, and age.
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