Cloning and sequencing of the cytoplasmic precursor to the α subunit of rat liver mitochondrial succinyl-CoA synthetase

(cDNA/ λ gt11/dideoxy sequencing/protein translocation/protein evolution)

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ABSTRACT Succinyl-CoA synthetase [succinate-CoA ligase (GDP-forming); EC 6.2.1.4] of rat liver, an $\alpha\beta$ dimer, is a component of the enzymology of the tricarboxylic acid cycle and functions within the mitochondrial matrix. We have isolated and determined the sequence of a cDNA clone containing the coding sequence of the cytoplasmic precursor to the α subunit of this enzyme together with stretches of nontranslated sequence at the 5' and 3' ends. The translated amino acid sequence indicates the presence of a 27-residue N-terminal signal sequence for mitochondrial targeting. The amino acid sequence of the mature α subunit shows an extraordinary degree of homology to the α subunit of *Escherichia coli* succinyl-CoA synthetase, with >70% of the residues identical. This suggests that the fundamental differences in the quaternary structures and catalytic functions of the mammalian and bacterial enzymes must be attributable to differences in the β subunits. mRNA that hybridizes to the cloned DNA is ≈ 1800 nucleotide residues in length, confirming that each of the two subunits is encoded separately and does not arise by proteolysis of a primary gene product containing both subunits of the mature protein.

Although the route and mechanism of protein import into mitochondria are becoming well understood as the result of investigation in many laboratories throughout this decade (e.g., see refs. 1–4 and references therein), a subject of awakening interest is the process by which monomeric polypeptide chains become assembled to form homologous or heterologous multisubunit protein molecules within the protein-dense environment of the mitochondrial matrix (1, 5). Related to this process is the formation of organized multienzyme structures ("metabolons") within the matrix as the result of specific interactions between enzyme molecules that catalyze separate but neighboring reactions on mitochondrial metabolic pathways such as the tricarboxylic acid and urea cycles (6, 7).

Succinyl-CoA synthetase [succinate-CoA ligase (GDP-forming); EC 6.2.1.4] in many ways represents an ideal system for the study of assembly of multisubunit structures within the mitochondrial matrix. This enzyme catalyzes the substrate level phosphorylation step of the tricarboxylic acid cycle:

It is known to be made up of two subunit types: the α subunit ($M_r \approx 30,000$) contains the histidine residue that is phosphorylated in the course of catalysis together with the binding site for the nucleotide substrate* (9), and the β subunit ($M_r \approx 40,000$) contains the binding site for succinate and CoA

(for a review, see ref. 10). The application of active enzyme centrifugation techniques (11) has established that the active form of the enzyme from *Escherichia coli* is an $\alpha_2\beta_2$ tetramer, whereas that of the mammalian mitochondrial enzyme is a nonassociating $\alpha\beta$ dimer. The genes encoding the subunits of the *E. coli* enzyme have been sequenced and their organization in the chromosome has been elucidated (12, 13), but until now the genetic organization, biosynthesis, transport, and assembly of the mammalian mitochondrial enzyme have not been investigated. In this paper, we describe the first phases of such a study, including the cloning and sequencing of cDNA encoding the α subunit of rat liver succinyl-CoA synthetase.[†] We also show that the translated amino acid sequence shows a remarkable degree of identity to that of the α subunit of the *E. coli* enzyme.

MATERIALS AND METHODS

Materials. Succinyl-CoA synthetase from porcine heart mitochondria was isolated as described by Wolodko *et al.* (11), and this was used for the production of antibodies in rabbits.[‡] All restriction enzymes were obtained from Boehringer Mannheim. A rat liver cDNA library in λ gt11 was kindly provided by M. Mueckler (Massachusetts Institute of Technology, Cambridge, MA). Horseradish peroxidase–goat anti-rabbit IgG was obtained from Bio-Rad as were reagents for antibody visualization.

Cloning and Sequencing. A rat liver cDNA library in λ gt11 was screened for the expression of fusion proteins that were immunologically reactive to succinyl-CoA synthetase antibodies, essentially as described by Huynh *et al.* (15), but by using horseradish peroxidase–goat anti-rabbit IgG instead of ^{125}I -labeled protein A to detect positive plaques. Nine positive clones were isolated that contained inserts ranging in size from ≈ 500 to ≈ 950 base pairs (bp). The restriction endonuclease digestion patterns indicated that these fell into two groups, suggesting that some contained coding sequence for the α subunit and some contained sequence for the β subunit. The clone containing the largest insert (λ SCS8) was selected for further study. The DNA insert from λ SCS8 was excised with *EcoRI* and ligated into the *EcoRI* site of M13mp18 (16). This was then partly sequenced by the dideoxy chain-termination method (17), and the translated

*The enzyme from bacterial and plant sources prefers adenine nucleotides, whereas that from mammalian mitochondria has been generally considered to be specific for guanine nucleotides. Recently, however, adenine nucleotide-specific isozymes have been demonstrated in mammalian systems (8).

[†]The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03621).

[‡]Ball and Nishimura (14) have demonstrated the presence of common antigenic determinants on pig heart and rat liver succinyl-CoA synthetases.

amino acid sequence was found to have very strong homology to the sequence of the α subunit of *E. coli* succinyl-CoA synthetase (see *Results and Discussion*). *Bam*HI and *Pst* I were used alone and in combination to produce fragments in both orientations for sequencing, and nested deletions were prepared by using exonuclease III (18) to complete the sequencing of the λ SCS8 insert. To obtain full-length clones, the λ SCS8 insert was nick-translated and this was used to probe plaques from the original λ gt11 library by hybridization at high stringency (18). Three clones containing inserts of 1.0–1.7 kilobases (kb) were thus isolated. The insert in one of these, λ SCS19, was sequenced by the same methods and by the use of chemically synthesized oligonucleotides. This was found to span the complete coding sequence for the precursor to the α subunit together with 5' and 3' untranslated sequences (see below).

RNA Transfer Blot Analysis. Poly(A)⁺ RNA from rat liver was prepared by standard methods (19). Preparations were denatured by heating at 68°C for 15 min in the presence of 50% deionized formamide/6% formaldehyde, subjected to electrophoresis in 1% agarose containing 0.6% formaldehyde, 20 mM 4-morpholinepropanesulfonic acid (pH 7.0), 5 mM sodium acetate, and 1 mM EDTA, and electrophoretically transferred to a nylon membrane (Hybond N, Amersham). The λ SCS8 950-bp insert was labeled by nick-translation (18) and 0.1 μ g (10⁷ dpm) was hybridized to the blot at 42°C in hybridization buffer (50% formamide/50 mM Tris-HCl, pH 7.5/0.5 M NaCl/1% NaDodSO₄/0.1% sodium pyrophosphate/0.2% bovine serum albumin/0.2% polyvinylpyrrolidone/0.2% Ficoll/10% dextran sulfate/100 μ g of salmon sperm DNA per ml). The blot was washed in 0.3 M NaCl/0.04 M Tris-HCl, pH 7.8/2 mM EDTA/0.1% NaDodSO₄ at 65°C, and the RNA that hybridized with the labeled probe was detected by autoradiography with intensifying screens.

RESULTS AND DISCUSSION

Clone Isolation and Identification. The initial approach taken was to screen a rat liver cDNA library in the expression vector λ gt11 (15) by using antibodies produced with intact (i.e., $\alpha\beta$) succinyl-CoA synthetase. This resulted in the isolation of nine clones, all of which produced β -galactosidase fusion proteins that were strongly immunoreactive. The one containing the largest insert ($\approx$ 950 bp, λ SCS8) was selected for further study. Preliminary sequencing of restriction fragments of its insert revealed a striking degree of homology of the translated amino acid sequence to regions of the α subunit of *E. coli* succinyl-CoA synthetase (see below), thus establishing that the insert of λ SCS8 contained a large portion of the structural gene of the α subunit of the rat liver enzyme. This insert was then labeled by nick-translation and used as a probe for the selection of further clones from the λ gt11 library. Those so isolated included λ SCS19, which contained the entire coding sequence for the precursor to the α subunit together with untranslated sequences.

Size of mRNA. Before this study was undertaken, no information was available concerning the location or organization of the genes for the subunits of succinyl-CoA synthetase. Furthermore, although all preparations of the enzyme have indicated the presence of two subunit types, it had not been established that these represent discrete gene products. For example, it might be argued that the appearance of two subunit types could be the result of a persistent proteolytic artifact or of specific *in vivo* proteolysis of a precursor polypeptide that contained both of the apparent subunit types. These possibilities have now been ruled out by the use of RNA transfer blot analysis using the labeled λ SCS8 insert as a probe. The results indicate that the size of the mRNA

for the α subunit of succinyl-CoA synthetase contains $\approx$ 1800 nucleotide residues (Fig. 1). Since this size is expected for mRNA for a protein of the size of the α subunit, the data thus establish that the subunits are encoded separately and do not arise by proteolysis of a primary gene product containing both subunits of the mature protein. We conclude that precursors to the α and β subunits are translocated separately into the mitochondrial matrix where assembly of the mature dimer occurs.

Nucleotide and Amino Acid Sequences. Fig. 2 shows the complete nucleotide sequence of the λ SCS19 insert together with the translated amino acid sequence for the α subunit of succinyl-CoA synthetase that it encodes. The open reading frame (nucleotide residues 491–1489) encodes the cytoplasmic precursor to the α subunit, and the 3' untranslated sequence includes an AATAAA consensus sequence (nucleotide residues 1663–1668) that normally occurs some 15–30 nucleotides upstream from the cleavage and poly(A) site (20–22), suggesting that polyadenylation of the mRNA may follow closely downstream from the 3' end of the fragment. The translated amino acid sequence of the open reading frame is aligned, virtually without gaps, with the amino acid sequence of *E. coli* α subunit in Fig. 3, showing 70% identity, plus extensive additional homology, between the two polypeptides. Also shown in Fig. 3 is the sequence of the 19 residues at the N terminus of the α subunit from pig heart obtained by sequential Edman degradation. This sequence is very highly homologous (14/19 identical) to a stretch near the N terminus of the rat liver α precursor, from which we infer that the N terminus of the mature rat liver subunit is as shown. Residues –1 to –27 thus represent the signal sequence for the translocation of the precursor from the cytoplasm to the mitochondrial matrix. This region of the polypeptide is typical of mitochondrial signal sequences in its length, hydrophobicity, high content of basic and serine residues, and absence of acidic residues (see ref. 2 and citations therein). Plotting of the 27 residues of the signal sequence on a "helical wheel" shows that an α -helix formed by such a sequence would be amphipathic, with all five positive charges clustered on one face. Potential for formation of amphipathic helices such as this is characteristic of mitochondrial signal sequences (23, 24).

Fig. 4 shows a plot of indices for hydrophobicity and α -helicity for the precursor calculated with the use of amino acid attributes tabulated by Bacon and Anderson (25). The M_r calculated from the sequence of the mature subunit is 31,630.

FIG. 1. Transfer blot analysis of rat liver poly(A)⁺ mRNA. ³²P-labeled λ SCS8 DNA was used to probe the nylon membrane blot. Samples are as follows. Lane 1, 6 μ g of mouse T-cell mRNA (control); lane 2, 6 μ g of rat liver mRNA (preparation no. 1); lane 3, 12 μ g of rat liver mRNA (preparation no. 1); lane 4, 12 μ g of rat liver mRNA (preparation no. 2).

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10      20      30      40      50      60      70      80      90      100
GCTCCTACTG GGCCTGACTT TGCTGGTGAG CTCCTGCGAA GCTTTGGTGT GCTTTGAGTG TGGCAACCTC AATTCTATGG GGATTGTGAA CTTTAGAACA

110     120     130     140     150     160     170     180     190     200
GCTGTATGTT ACGCTCATCC TGGTGAGGTT TGTGCCTCAG TTTTAACCTA TAAAGATGGA AAATTTGTAT ATGGAACCA GAGCTGTGCT GAGTGTCCG

210     220     230     240     250     260     270     280     290     300
GGAGGACTGT TGAGCATGGA AGTCTGATAG TATCAACAAA CTGTTGCTCA GCCACCTCTT TCTGCAATAT AGTACGTCCC TGATACTTAA TTGTATGGAA

310     320     330     340     350     360     370     380     390     400
GGATTTTAGT GCTCTTTACT GAGTCTTGAC ACTGGAGCAT GCCAGAAAGA ATTATCTACT GACCGTGTCT GGAGAACGCA CTAAGATTTC TACATCATGT

410     420     430     440     450     460     470     480     490
TTTCTGACTA CGTGATATC ATATCATATG ATCTTCAGTG ATCTGTAATA AATTGAAGGC CTTACAAATT TTGCGGCTGC CACTGCAACC

505     520     535     550     565
ATG GTC TCC GGC AGC AGC GGC CTT GCC GCC GCC CGC CTG CTG TCG CGC ACC TTC CTC CTG CAA CAG AAT GGA ATA
MET Val Ser Gly Ser Ser Gly Leu Ala Ala Ala Arg Leu Leu Ser Arg Thr Phe Leu Leu Gln Gln Asn Gly Ile

580     595     610     625     640
CGG CAC GGG TCT TAC ACA GCC TCT CGG AAA AAT ATC TAT ATT GAT AAA AAT ACG AAG GTT ATT TGC CAG GGT TTC
Arg His Gly Ser Tyr Thr Ala Ser Arg Lys Asn Ile Tyr Ile Asp Lys Asn Thr Lys Val Ile Cys Gln Gly Phe

655     670     685     700     715
ACA GGC AAG CAG GGT ACC TTT CAC AGC CAG CAG GCT TTG GAG TAC GGC ACC AAA CTC GTG GGA GGA ACC ACT CCA
Thr Gly Lys Lys Gln Gly Thr Phe His Ser Gln Gln Ala Leu Glu Tyr Gly Thr Lys Leu Val Gly Gly Thr Thr Pro

730     745     760     775     790
GGG AAA GGG GGC AAG AAG CAC CTG GGC TTA CCT GTC TTT AAT ACT GTG AAG GAA GCC AAA GAG AAA ACA GGA GCG
Gly Lys Gly Gly Lys Lys His Leu Gly Leu Pro Val Phe Asn Thr Val Lys Glu Ala Lys Glu Lys Thr Gly Ala

805     820     835     850     865
ACA GCT TCT GTC ATT TAT GTC CCT CCT CCT TTC GCT GCT GCC GCC ATC AAT GAA GCA ATT GAC GCA GAG ATT CCC
Thr Ala Ser Val Ile Tyr Val Pro Pro Phe Ala Ala Ala Ile Asn Glu Ala Ile Asp Ala Glu Ile Pro

880     895     910     925     940
TTG GTT GTG TGC ATT ACT GAA GGT ATT CCG CAG CAG GAC ATG TTG CGG GTC AAG CAC AAG CTG ACA CGC CAG GGG
Leu Val Val Cys Ile Thr Glu Gly Ile Pro Gln Gln Asp MET Leu Arg Val Lys His Lys Leu Thr Arg Gln Gly

955     970     985     1000     1015
AAG ACG AGG CTA ATT GGG CCA AAC TGC CCC GGT ATC ATC AAC CCT GGA GAA TGC AAA ATC GGC ATC ATG CCT GGC
Lys Thr Arg Leu Ile Gly Pro Asn Cys Pro Gly Ile Ile Asn Pro Gly Glu Cys Lys Ile Gly Ile MET Pro Gly

1030    1045    1060    1075    1090
CAC ATT CAC AAG AAA GGA AGA ATA GGT ATC GTG TCC AGG TCC GGT ACT CTG ACT TAT GAA GCA GTT CAC CAA ACA
His Ile His Lys Lys Gly Arg Ile Gly Ile Val Ser Arg Ser Gly Thr Leu Thr Tyr Glu Ala Val His Gln Thr

1105    1120    1135    1150    1165
ACC CAA GTT GGA TTG GGG CAG TCC TTG TGT ATT GGC ATT GGA GGT GAC CCT TTT AAT GGG ACT AAT TTT ATT GAT
Thr Gln Val Gly Leu Gly Gln Ser Leu Cys Ile Gly Ile Gly Gly Asp Pro Phe Asn Gly Thr Asn Phe Ile Asp

1180    1195    1210    1225    1240
TGC CTT GAC GTC TTC CTG AAG GAT CCA GCT ACA GAA GGC ATC GTA CTG ATT GGT GAA ATT GGT GGT CAC GCT GAA
Cys Leu Asp Val Phe Leu Lys Asp Pro Ala Thr Glu Gly Ile Val Leu Ile Gly Glu Ile Gly Gly His Ala Glu

1255    1270    1285    1300    1315
GAA AAT GCC GCG GAG TTT CTG AAG GAG CAT AAC TCA GGT CCA AAG GCC AAG CCT GTA GTG TCC TTC ATT GCT GGT
Glu Asn Ala Ala Glu Phe Leu Lys Glu His Asn Ser Gly Pro Lys Ala Lys Pro Val Val Ser Phe Ile Ala Gly

1330    1345    1360    1375    1390
ATA ACT GCT CCT CCT GGC AGA AGG ATG GGC CAC GCA GGG GCG ATT ATT GCT GGA GGA AAA GGT GGC GCC AAA GAG
Ile Thr Ala Pro Gly Arg Arg MET Gly His Ala Gly Ala Ile Ile Ala Gly Gly Lys Gly Gly Ala Lys Glu

1405    1420    1435    1450    1465
AAG ATC TCT GCT CTT CAG AGT GCG GGT GTA ATT GTC AGC ATG TCC CCC GCA CAG CTG GGA ACC TGC ATG TAT AAG
Lys Ile Ser Ala Leu Gln Ser Ala Gly Val Ile Val Ser MET Ser Pro Ala Gln Leu Gly Thr Cys MET Tyr Lys

1480    1502    1512    1522    1532    1542    1552
GAA TTT GAA AAG AGG AAG ATG CTT TGA AGGACACGAC ATGAATTTTA AGGCTGTGGA GGAAATCTCA CAGACTGGAG AGCCCGTCCA
Glu Phe Glu Lys Arg Lys MET Leu

1562    1572    1582    1592    1602    1612    1622    1632    1642    1652
GACTGCAGCC CAGGCACCTG ACCCTGGTCT CTCAGTACAG GGGAAAGTCCA GGCATGTTTT AGAATGTCCT AAATTGAGCT ATTTTCACIT ACTGTGTAAC

1662    1672    1682
AGAGACAGAT AATAAATCTA TCATTGATT TGAAAAAAA

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FIG. 2. Sequence of λ SCS19 insert. The derived amino acid sequence for the α subunit of succinyl-CoA synthetase is also shown.

Further comment on specific regions of the sequences of rat liver and *E. coli* α subunits is appropriate. The active site histidine (His-258) that is phosphorylated in the course of catalysis is indicated by the asterisk in Fig. 3. The surrounding sequence, first elucidated by isolation and sequencing of a phosphohistidine-containing dodecapeptide from the *E. coli* enzyme (26), is contained within a stretch of 14 identical

residues. Buck *et al.* (12) have identified three potential regions of the *E. coli* α subunit, which, by analogy with other kinase structures, might be part of the nucleotide binding domain of this subunit. Examination of the rat liver sequence shows that two of these (amino acid residues 42–51 and 208–219) have almost identical sequences to their *E. coli* counterparts, consistent with an important functional role.

		Rat Liver Signal Sequence	MVSGSSGLAARLLSRTFLLQNGIRH -27
Pig heart	1	GSYTASGKHLVVDKNTIVF---	30
Rat Liver		GSYTASRKNIYIDKNTKVICQGF	TGKGFHSSQQALEYGTKLVGGTTPGKGGKK
<i>E. coli</i>	1	mSILIDKNTKVICQGF	TSQGFHSEQAIAYGTKMVGGVTPGKGGTT
	60	HLGLPVFNTVKEAKEKTGATASVIYVPPFAAA	INEAIDAEIPLVVCITEGI
Rat Liver		HLGLPVFNTVREAAATGATASVIYVPA	FFCKDSILEAIDAGIKLIITITEGI
<i>E. coli</i>			
	120	PQODMLRVKHLTRQKTRLIGPNC	PGIINPGECKIGIMPGHIHKGRIGIVS
Rat Liver		PTLDMLTVKVKLDEAG.VRMIGPNC	PGVITPGECKIGIQPGHIHKPGKVGIVS
<i>E. coli</i>			
	180	RSGTLTYEAVHQTTOVGLGQSLCIGIG	GGDPFNGTNFIDCLDVFLKDPATEGIV
Rat Liver		RSGTLTYEAVKQTTDYGFGQSTCVGIG	DDIPGSNFIIDILEMFEKDPQTEAIV
<i>E. coli</i>			
	240	LIGEIGGHAEEENAAEFLKEHNSGPKAK	PVVSFIAGITAPPGRMRMGHAGAI IAG
Rat Liver		MIGEIGGSAAEEAAAYIKEHVT. . .	KPVVGYIAGVTAPKGRMGHAGAI IAG
<i>E. coli</i>			
	270	GKGGAKEKISALQSAGVIVSMSPAQLG	TCMYKEFEKRMKL
Rat Liver		GKGTADKFAALEAGVKTVRSLADIGEAL	KTVLK
<i>E. coli</i>			

FIG. 3. Comparison of the amino acid sequences of the α subunits of rat liver and *E. coli* succinyl-CoA synthetases. The sequence of the bacterial subunit is taken from ref. 12. The bold lines indicate residues that are identical in the bacterial and mammalian enzymes, and the asterisk indicates the location of the active site phosphohistidine residue.

The third sequence identified by Buck *et al.*, corresponding to residues 95–102 in rat liver α subunit, has much reduced homology.

The degree of homology between the rat liver and *E. coli* α subunits is remarkable. As indicated above, 70% of the residues are identical, and the inclusion of conservative replacements would lift the degree of homology to almost 90%. Where similar comparisons have been made for other mitochondrial proteins, much lower degrees of homology have been found. For citrate synthase, which is an appropriate example because it also catalyzes a CoA-dependent step of the tricarboxylic acid cycle, sequence determination of the pig heart mitochondrial and *E. coli* enzymes has shown that only short stretches of sequence near the active site have high homology (27). The highest degree of sequence match that has been detected between eukaryotic amino acyl-tRNA synthetases (either cytoplasmic or mitochondrial) and their prokaryotic counterparts is 40% (28), but that match required several gaps. The extent of homology that we have found for the α subunit of succinyl-CoA synthetase is, to our knowledge, unprecedented. Despite

this, succinyl-CoA synthetases from bacterial and mammalian sources have distinct and well-documented differences in properties. For example, the active form of the enzyme from *E. coli* is known to be a nondissociating $\alpha_2\beta_2$ tetramer, whereas the mitochondrial enzyme is active as an $\alpha\beta$ dimer (11). Many lines of evidence (29–31) point to cooperative catalytic interactions between the two $\alpha\beta$ halves of the *E. coli* enzyme that are not possible in the mammalian version. It would be difficult to attribute these fundamental differences in properties of the two enzymes to the α subunits, since these are almost identical. It will thus be of considerable interest to determine the structure of the β subunit of the mammalian enzyme to learn if the high prokaryotic/eukaryotic homology extends to it.

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FIG. 4. Hydrophobicity (bold lines) and α -helicity (shaded areas) indices calculated for the amino acid sequence of the α subunit of rat liver succinyl-CoA synthetase. The arrow indicating the cleavage point divides the signal sequence (residues -27 to -1) from the mature subunit.

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