The mitochondrial carnitine carrier protein: cDNA cloning, primary structure and comparison with other mitochondrial transport proteins

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The amino acid sequence of the rat carnitine carrier protein, a component of the inner membranes of mitochondria, has been deduced from the sequences of overlapping cDNA clones. These clones were generated in polymerase chain reactions with primers and probes based on amino acid sequence information, obtained from the direct sequencing of internal peptides of the purified carnitine carrier protein from rat. The protein sequence of the carrier, including the initiator methionine, has a length of 301 amino acids. The mature protein has a modified α-amino group, although the nature of this modification and the precise position of the N-terminal residue have not been ascertained. Analysis of the carnitine carrier sequence shows that the protein contains a 3-fold repeated sequence about 100 amino acids in length. Dot plot comparisons and sequence alignment demonstrate that these repeated domains are related to each other and also to the repeats of similar length that are present in the other mitochondrial carrier proteins sequenced so far. The hydropathy analysis of the carnitine carrier supports the view that the domains are folded into similar structural motifs, consisting of two transmembrane α-helices joined by an extensive extramembranous hydrophilic region. Southern blotting experiments suggest that both the human and the rat genomes contain single genes for the carnitine carrier. These studies provide the primary structure of the mitochondrial carnitine carrier protein and allow us to identify this metabolically important transporter as a member of the mitochondrial carrier family, and the sixth of the members whose biochemical function has already been identified.

INTRODUCTION

The inner membranes of mitochondria contain a number of specific carrier proteins responsible for the transport of various metabolites, nucleotides and cofactors into and out of the matrix space (reviewed in [1]). Previously, 10 of these proteins have been purified to homogeneity from mammalian mitochondria (reviewed in [2]). One example is provided by the carnitine carrier (CAC), also known as the carnitine/acylcarnitine translocase, which has been isolated from rat liver mitochondria and has an apparent molecular mass of 32.5 kDa [3]. It catalyses the exchange of acylcarnitines of various lengths with carnitine across the mitochondrial membrane, and therefore plays a central role in the translocation of fatty acids via acylcarnitines into the mitochondrial matrix, where the acyl groups are released to be used for fatty acid oxidation. Recently, several patients with a deficiency of carnitine/acylcarnitine translocase have been described [4–6]. From the information available, CAC deficiency is among the most severe disorders of fatty acid oxidation and can be lethal at an early stage. Because of the importance of the CAC in the intermediary metabolism, the functional properties of this carrier have been extensively investigated both in intact mitochondria [7–13] and after purification and reconstitution into liposomes [3,14–18].

Amino acid sequences have been published for the ADP/ATP, phosphate, 2-oxoglutarate and citrate carriers [19–22], as well as for the uncoupling protein [23], which is an H^+ carrier present in mitochondria in brown fat. All these carriers have a similar molecular mass of approx. 30 kDa and have related primary structures. The polypeptide chains of each of these transporters are made up of three related segments, approx. 100 amino acid residues in length. Furthermore all of these repetitive elements could be folded into a similar structural motif consisting of two transmembrane α-helices joined by an extensive hydrophilic extramembrane loop (reviewed in [2]). The similarities in sequence and structure between the ADP/ATP translocase, the phosphate carrier and the uncoupling protein led to the generally accepted concept of the mitochondrial carrier family [20,23], which may have evolved from a common ancestor of approx. 100 amino acids. It was also suggested that the other mitochondrial carriers that have been biochemically characterized, but not yet sequenced, belong to the same carrier protein family [1,24] because they have structural and functional features in common with the established members. This assumption, the proof of whose validity would require a knowledge of their primary structures, was questioned for the CAC because this transporter functions by a Ping Pong reaction mechanism [17], whereas all the other mitochondrial carriers analysed so far in the reconstituted state follow a simultaneous (sequential) mechanism [2,25].

In this paper we report the primary structure of the mitochondrial CAC. Using polymerase chain reaction-amplified CAC cDNA fragments, we sequenced a cDNA that encodes the entire CAC. The sequence of this transporter, consisting of 301 amino acid residues, contains three related internal domains. Its tripartite structure, the similarity of its hydrophobic profile to those of the other mitochondrial carriers, and the clear sequence similarity to the other mitochondrial carrier sequences, including the 3-fold repetition of a characteristic sequence motif, allow us to assign the CAC to the mitochondrial carrier family.

Abbreviation used: CAC, carnitine carrier.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number X97831.

EXPERIMENTAL

Isolation of CAC

The protein was isolated from rat liver mitochondria, reconstituted into liposomes and assayed for transport activity as described previously [3,26].

Fragmentation and sequence analysis of CAC

The N-terminal sequence of a sample of purified carrier protein was examined in a pulsed-liquid protein sequencer (Applied Biosystems 477A) equipped with an on-line phenylthiohydantoin-amino acid analyser. A second sample of purified carrier was precipitated with a 20-fold excess of cold acetone for 4 h at −20 °C, washed twice with a mixture of acetone/H_2O (4:1), and solubilized in SDS/sample buffer [100 mM Tris/HCl (pH 6.8)/1 % (w/v) SDS/10 % (v/v) glycerol/100 mM dithioerythritol] by heating at 80 °C for 10 min. The CAC protein was then run on SDS/PAGE [3] and electrophoretically transferred to a poly(vinylidene difluoride) membrane (ProBlott; Applied Biosystems). The protein, detected by staining with Coomassie Blue, was excised, washed twice with methanol and subjected to Edman degradation in the pulsed-liquid protein sequencer. For internal sequence determinations, the purified CAC protein, precipitated with acetone and washed as described above, was solubilized in 70 % (v/v) formic acid and digested with a 20-fold molar excess of cyanogen bromide at room temperature in the dark [27]. After 13 h, the solution was diluted 1:10 with water and then freeze dried. The dried peptides, dissolved in SDS/sample buffer, were separated either by SDS/PAGE with the system of Schägger and von Jagow [28] or by reverse-phase HPLC on a C_{18} Nucleosil column in 0.1 % trifluoroacetic acid with a linear gradient of acetonitrile. In other experiments proteolysis of purified CAC with *Staphylococcus aureus* V8 proteinase (Boehringer) was performed on slices of SDS gel or *in vitro* by the method of Cleveland [29]. In the first case, purified CAC was subjected to SDS/PAGE [3]. After staining with Coomassie Blue, the bands were cut out and equilibrated with 100 mM Tris/HCl (pH 6.8)/12 % (v/v) glycerol/50 mM dithioerythritol/2 % (w/v) SDS (buffer A) for 10 min. These gel fragments were then inserted into the slots of a gel 1.5 mm thick and covered with the same buffer containing 20 % (v/v) glycerol, on top of which the solution of V8 proteinase was layered. The separating gel was 6 cm high and contained 16 % (w/v) polyacrylamide (acrylamide/bisacrylamide 30:0.8, w/w). The stacking gel was 2 cm high. After the samples had been stacked with a 4 mA constant current, the power was turned off for 3 h. Thereafter the peptides were separated in the separating gel with a 25 mA constant current. Proteolysis *in vitro* was performed on purified CAC after precipitation with acetone and washing. The pellet (dissolved in buffer A) was incubated with *S. aureus* V8 proteinase (protein/proteinase ratio 50:1) for 20 h at 25 °C. The reaction was stopped by boiling the samples for 10 min. The peptides were then separated by HPLC as described above. The peptides, separated either by SDS/PAGE or by HPLC, were subjected to Edman degradation in the pulsed-liquid protein sequencer.

Synthetic oligonucleotides

Oligonucleotides were synthesized in an Applied Biosystems model 320B DNA synthesizer. Some were used as primers in polymerase chain reactions (see Table 1) and others as hybridization probes to investigate the products of these reactions. Forward and reverse primers were made with *Eco*RI and *Hin*dIII linkers respectively on their 5′ ends. Some unique oligonucleotides 17 bases long were employed as primers in the sequencing reaction. The purification, radiolabelling and use as hybridization probes of synthetic oligonucleotides have been described previously [30].

Amplification of CAC cDNA by polymerase chain reaction

Polymerase chain reactions were conducted as described before [31] in a Perkin-Elmer DNA thermal cycler, model 480. The 70 μl reaction mixtures contained 1 ng of rat liver cDNA template (Clontech) with primers added to a final concentration of 0.5 μM. After an initial incubation at 94 °C for 2 min without Amplitaq polymerase, a three-step cycle that included denaturation at 94 °C (30 s), annealing at 4 °C below the minimum dissociation temperature and extension at 72 °C (90 s) was repeated 30 times, followed by a final extension at 72 °C (7 min). After addition of fresh enzyme, a further 30 identical reaction cycles were performed. To extend the sequence to the 5′ end an adaptor-ligated double-stranded rat liver cDNA (Clontech) was used as template in a touchdown polymerase chain reaction [32]. In these

Table 1 Synthetic oligonucleotides related to the carnitine carrier protein that were employed as primers and probes in polymerase chain reactions with rat liver cDNA as template

In the first column, F and R denote forward and reverse primers, a and b denote nested primers, and P denotes oligonucleotide probes. Oligonucleotides $6P_{ex}$ and $7P_{ex}$ were primers used to extend existing sequences. Unique oligonucleotides were based on sequences of partial cDNA clones generated by polymerase chain reactions.

Oligonucleotide	Protein sequence used in design oligonucleotides	Sequence	Complexity
1F, 2P and 4P	YPQLFTA	TAYCCNCARYTNTTYACNGC	1024
1R and 3P	IMTPGE	TCNCCNGGNGTCATDAT	192
1P	GVFTTG	GGNGTNTTYACNACNGG	512
2F	MFAVCF	ATGTTYGCNGTNTGYTT	64
2R and 4R	GMLSGVFTTG	TCCTGTGGTGAACACACCAGATAACATCC	1
3F	GMLSGVFTTG	GGATGTTATCTGGTGTGTTCACCACAGGA	1
3R		$(A)_{21}$	1
4Fa		CCATCCTAATACGACTCACTATAGGGC	1
4Fb		ACTCACTATAGGGGCTCGAGCGGC	1
$6P_{ex}$		TGGCTTTGAAATTCCGAT	1
$7P_{ex}$		AAGTTGGGGGCAATCCA	1

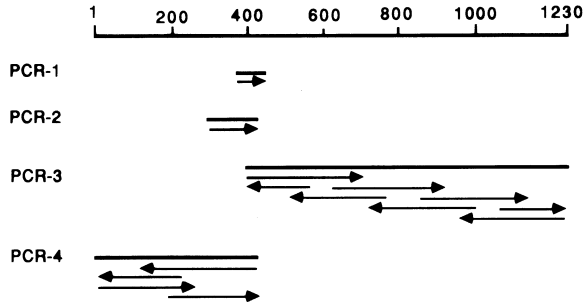

Figure 1 Generation by polymerase chain reactions and sequence analysis of clones encoding the CAC from rat liver

PCR-1 to PCR-4 are the cDNA segments generated by polymerase chain reactions (for details see the text). The heavy horizontal lines are proportional to the lengths of these cDNA segments; the arrows represent the directions and the extents of the determined DNA sequences. The scale is in bases. The primers and the probes are listed in Table 1.

experiments the three-step cycle was modified as follows: denaturation at 94 °C, annealing at 70 °C (2 min) and extension at 72 °C (2 min), five times. Afterwards, the cycle was repeated five times at an annealing temperature of 65 °C and 20 further times at an annealing temperature of 60 °C. The reaction products were analysed on 2% (w/v) agarose gels. The DNA fragments were transferred to Hybond-N membranes (Amersham), cross-linked to the membranes by irradiation with UV, and hybridized with radioactively labelled synthetic oligonucleotides at 5 °C below the minimum dissociation temperature. The fragments that hybridized with the probes were recovered from the gel by the Gene Clean procedure (Bio 101), the *Eco*RI and *Hin*dIII sites in the linker sequences were hydrolysed, and the products were cloned into M13mp18 and M13mp19 vectors.

DNA sequence analysis

DNA sequences were determined by the modified dideoxy chain termination method [33] with T7 DNA polymerase (Sequenase; U.S. Biochemicals). Sequencing reactions were primed with either the 17-mer universal primers or the primers used in the polymerase chain reactions. Other 17-mer synthetic primers were used to extend existing sequences. Both DNA strands were sequenced completely. To avoid sequence errors introduced by the polymerase chain reaction, at least three independent M13-isolated clones were sequenced from each reaction. When an ambiguity was observed, further clones were sequenced. Compressions were resolved by the use of dITP instead of dGTP in sequencing reactions. Databases were compiled and analysed with the computer program Autoassembler (Perkin-Elmer).

Hybridization with rat and human genomic DNA

Rat and human genomic DNA species were prepared from liver and peripheral blood respectively [34]. The cDNA probe was prepared by random priming labelling of the purified product of PCR-3 (see Figure 1). Samples of each DNA (10 μg) were digested with one of the restriction endonucleases *Eco*RI, *Hin*dIII and *Xho*I for 6 h at 37 °C. The DNA fragments were separated on 0.7% agarose gel, transferred to Hybond-N membranes [35] overnight and cross-linked to the membranes by irradiation with UV. Hybridization was performed with the cDNA probe as described previously [21]. The filters were washed with 6 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1% SDS once at room temperature for 1 min, twice at 65 °C for 25 min, and then with 3 × SSC/0.1% SDS at 65 °C for a further 25 min. Finally they were exposed to X-ray film at −80 °C for 5 days.

RESULTS AND DISCUSSION

Partial protein sequence analysis of the CAC protein

No N-terminal sequence was detected when samples of the intact carrier protein were subjected to Edman degradation. This means that the purified carrier protein has a modified α-amino group, although the nature of this modification is unknown. Partial internal protein sequences were determined by analysis of *S. aureus* V8 proteinase and cyanogen bromide peptides isolated from digests of purified preparations of the carrier. The results of these experiments are summarized in Table 2.

Isolation and sequence analysis of cDNA clones encoding the CAC protein

To isolate the cDNA encoding the CAC we adopted a strategy based on minimal protein sequence information and the use of the polymerase chain reaction. In a first round of polymerase chain reactions, a short cDNA was generated by using degenerated forward and reverse primers based on the N- and C-terminal extremities of the 25-residue peptide LTYPQLFTAG-MLSGVFTTGIMTPGE (Table 2). These forward and reverse primers (1F and 1R of Table 1 and Figure 2) were 20 and 17 bases in length (plus appropriate linker sequences) respectively, with complexities of 1024 and 192 respectively. The products of the reaction were cloned and tested for hybridization with the degenerated probe 1P based on the central region of the above-mentioned protein sequence. This probe was 17 bases long and its complexity was 512. The only cDNA product (PCR-1 of Figure 1) that hybridized with the probe encoded a segment of 23 amino acids of the known protein sequence, the information of which was used for this experiment. However, because mixed primers were used in the polymerase chain reaction, only the central part of the sequence between the two primers was accurate. Therefore this central sequence was used as the basis of further experiments to generate the complete cDNA. First, we performed another polymerase chain reaction experiment in which the unique forward primer 3F (Table 1 and Figure 2) was used with the degenerated reverse primer TACAADCGNCAN-

Table 2 Partial protein sequences determined on the CAC protein or on digestion products

Partial internal CAC sequences were obtained with *S. aureus* V8 proteinase and cyanogen bromide digests of the purified CAC, as described in the Experimental section. Amino acid residues are depicted in the single-letter code. Residues in parentheses indicate data assigned with lower confidence. The presence of an X indicates a cycle in which no amino acid could be determined.

Sample	Determined sequence	Position in protein
Intact protein	None	
S. aureus V8 digest	LTYPQLFTAGMLSGVFTTGIMTPGE	108–132
	LSVP(R)VLVAG	207–216
	VPASG	180–184
	LIREEGVT	256–263
Cyanogen bromide digest	FAVXFFGFGLGKRLQQ	86–101
	LSGVFTTGIMTPGE	119–132
	YFMTYEXLKNXF	186–197
	TYE(H)LKNLF	189–197

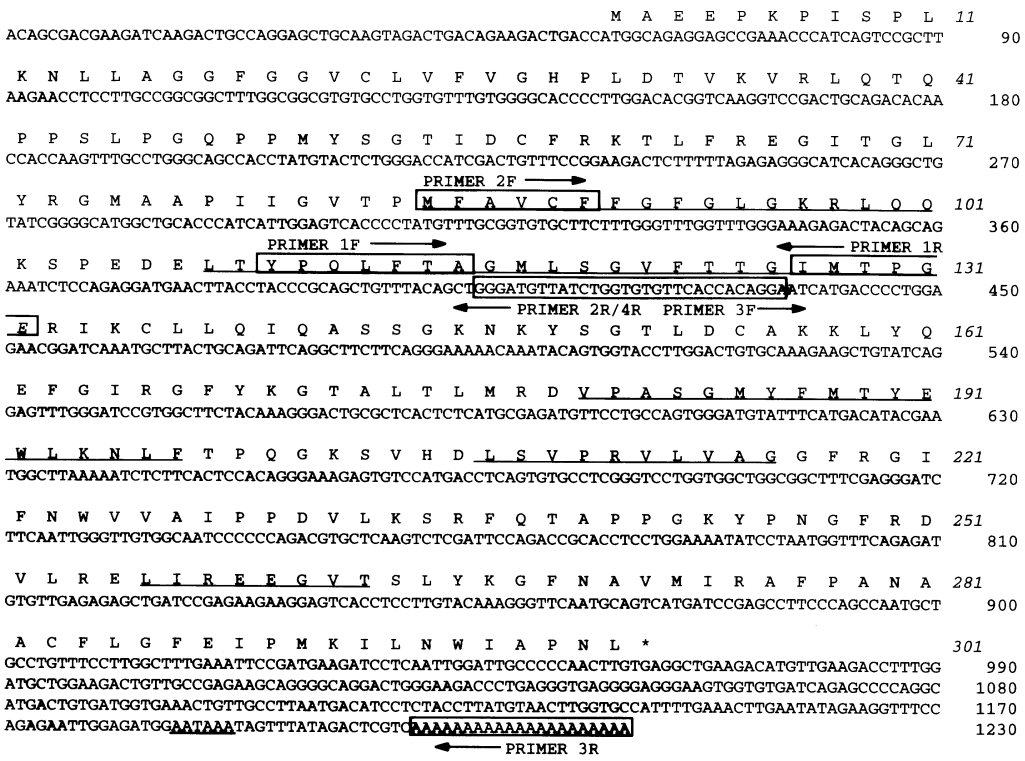

Figure 2 Compiled sequence of the cDNA encoding the CAC from rat liver mitochondria and the deduced amino acid sequence of the transporter

The deduced protein sequence is numbered from 1 to 301. An asterisk denotes the stop codon. The precise position of the modified N-terminal residue is unknown. Protein sequences determined by sequence analysis of fragments isolated from V8 proteinase and cyanogen bromide digests of the purified CAC protein are underlined. The boxed protein sequences were used to design mixtures of oligonucleotides employed as primers in polymerase chain reactions. The boxed nucleotide sequences are those of unique primers. Horizontal arrows pointing to the left or right indicate that the primers were synthesized as the sequence shown or as its complement respectively. The underlined sequence AATAAA is a potential polyadenylation signal.

ACDAA, based on another available protein sequence, i.e. MFAVCF (Table 2). However, the products obtained in this experiment did not hybridize with the mixed probe ATHATG-ACNCCNGGNGA. In contrast, by using the unique reverse primer 2R (Table 1 and Figure 2) and the mixed forward primer 2F (based on the protein sequence MFAVCF), a product (PCR-2; Figure 1) was generated that hybridized with the degenerated probe 2P, based on the protein sequence YPQLFTA. The sequence of the cDNA for the CAC was completed in two further experiments of polymerase chain reaction (PCR-3 and PCR-4 in Figure 1). The sequence generated in PCR-1 was extended in the 3′ direction by priming from the poly(A) tail (PCR-3; Figure 1). Three bands were obtained, among which only that of 824 bp hybridized with the degenerated probe based on the protein sequence IMTPGE (the C-terminal portion of the first peptide of Table 2). Finally, in experiment PCR-4, the sequence produced in PCR-1 was extended in the 5′ direction by a touchdown polymerase chain reaction (see the Experimental section) with two nested forward adaptor primers (4Fa and 4Fb) and the unique reverse primer 4R (Table 1). A single band of 435 bp was obtained, cloned and sequenced (Figures 1 and 2). This sequence overlapped that produced in PCR-2 and extended beyond the start codon (Figure 2). The complete cDNA sequence, shown in Figure 2, is compiled from the overlapping cDNA clones and has been completely determined in both directions (see Figure 1). It is 1230 nt in length with 5′ and 3′ untranslated regions of 57 and 270 bp respectively. The sequence is terminated by a tail of A residues separated by a sequence of 17 nt from the preceding sequence AATAAA, a typical signal for poly-adenylation of RNA [36].

The amino acid sequence of CAC

As shown in Figure 2, the compiled cDNA sequence encodes a protein of 301 amino acid residues. The initiation codon ATG at nt 58–60 can be identified with some certainty because a potential termination codon, TAG, is found in frame at nt 37–39. Because the N-terminus of the purified protein was found to be modified in an unknown way, and because the possibility cannot be excluded that the initial translation product might have undergone some post-translational proteolytic processing, the modified N-terminal residue of the mature protein could not be identified. The molecular mass of the protein as calculated from the sequence of the unmodified protein was 33132 Da, which is in good agreement with the value of 32500 Da estimated for the rat liver protein by SDS/PAGE [3]. More importantly, all amino acid sequences that we have chemically determined with digests of the purified CAC (Table 2, and underlined sequences of Figure 2) are present in the complete deduced amino acid sequence of the CAC protein (Figure 2). The polarity of the CAC, calculated from the amino acid composition [37], is 35.2%, i.e. lower than those of the other biochemically characterized mitochondrial carriers. These range from 35.9% for the phosphate carrier to 41.8% for the uncoupling protein (see Table III in [22]). The CAC contains 35 basic residues (H + K + R) and 20 acidic ones (D + E) and

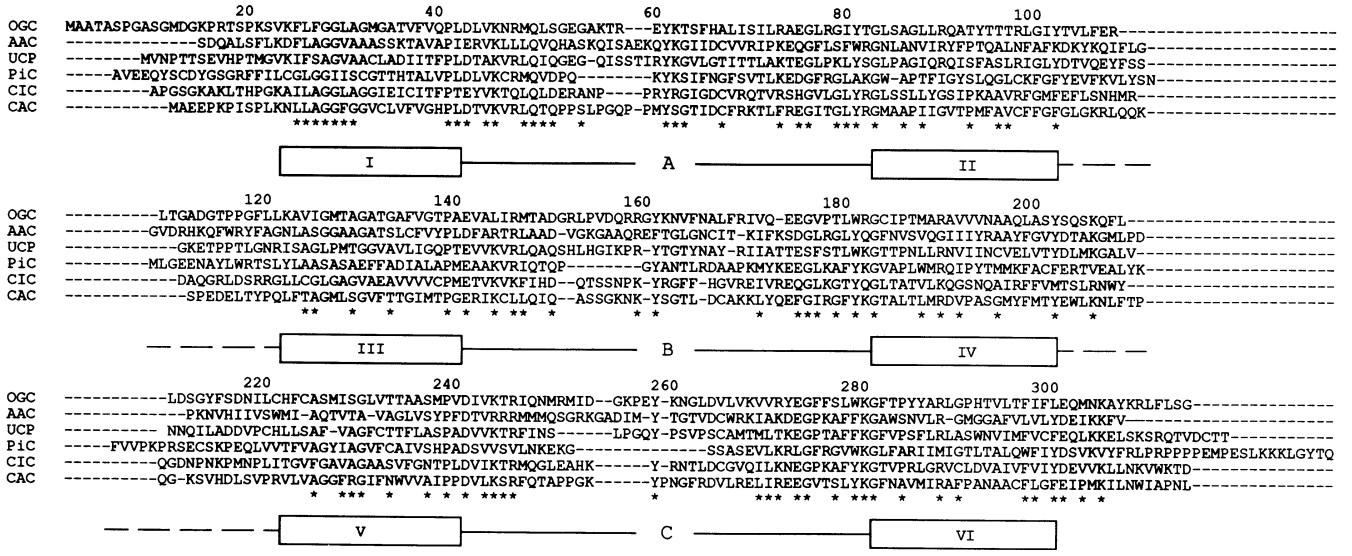

Figure 3 Alignment of the CAC repetitive sequence domains with those present in the other functionally characterized mitochondrial carriers sequenced so far

The alignments are based on dot plot comparisons. Numbers refer to the 2-oxoglutarate carrier sequence. The asterisks indicate residues that are identical or conservatively substituted in at least five out of the six sequences within a given domain. I–VI represent hydrophobic regions that might be folded into transmembrane α-helices. The transmembrane segments are linked by extensive hydrophilic regions (A, B and C) and the three repeats by shorter stretches of hydrophilic amino acids indicated by the dotted lines. Abbreviations: OGC, oxoglutarate carrier; AAC, adenine nucleotide carrier; UCP, uncoupling protein; PiC, phosphate carrier; CIC, citrate (tricarboxylate) carrier.

displays a positive net charge intermediate between the uncoupling protein and the phosphate carrier (+9 and +11 respectively) and the oxoglutarate, citrate and ADP/ATP carriers (+15, +17 and +18 respectively). Finally, the primary sequence of the CAC contains six cysteine residues, as previously determined by titration of the SDS-denatured purified carrier with 5,5′-dithiobis-(2-nitrobenzoic acid) [18].

Repetitive sequences in the CAC protein and comparison with the five functionally characterized mitochondrial carrier proteins sequenced so far

A dot plot comparison of the sequence of the CAC with itself (result not shown) reveals that this protein has a tripartite structure made of three tandem repeats of approx. 100 amino acid residues. Moreover, the three repeated domains of the CAC are related to those present in the other functionally characterized mitochondrial carriers [20–23,38].

Figure 3 shows the alignment of the 18 repetitive domains that constitute the six functionally characterized mitochondrial carrier proteins sequenced so far. The sequence of the CAC contains 33 residues that are either identical or conservatively substituted in at least 12 of the 18 repeats. Considering the alignment of the first, second or third repetitive element separately, 91 positions of the CAC are found at which amino acid residues are either identical or conservatively substituted in at least five of the six mitochondrial carriers. More importantly, the CAC displays the sequence motif characteristic of the mitochondrial carriers P-h-D/E-X-h-K/R-X-R/K-(20–30 residues)-D/E-G-(four residues)-a-K/R-G (where h represents hydrophobic and a represents aromatic), which is present in each of the three domains. These results clearly indicate that the CAC is a member of the mitochondrial carrier family.

Homology of the CAC protein sequence with other protein sequences

A comparison of the CAC sequence with those in the EMBL Nucleotide Sequence Database revealed significant similarity not only with the functionally characterized mitochondrial carriers sequenced so far (see above), but also with some proteins encoded by the *Saccharomyces cerevisiae* and *Caenorhabditis elegans* genomes, as well as with a protein associated with Grave's disease, a protein from *Oxytricha fallax*, the maize brittle 1 protein and the PMP47 from *Candida boidinii* (reviewed in [2,24,39]), which therefore should also be considered members of the mitochondrial carrier family, although their function is not yet known. In particular, the yeast genome (of which the sequence has now been completed) contains more than 30 putative members of the mitochondrial carrier family. Among them is a putative homologue of the rat CAC (located on chromosome XV), which displays a 28.9% identity over 301 amino acids and a 43.1% similarity if conservative substitutions are allowed. Apart from these proteins, the computer search revealed no significant sequence similarity with any known protein.

Hydrophobicity analysis and proposed transmembrane topology of the CAC

The structural relation between the CAC protein and the five biochemically characterized mitochondrial carriers sequenced so far is also revealed by the similarities in their hydrophobic profiles (results not shown). These profiles can be interpreted as indicating that the carriers contain six hydrophobic segments (denoted as I–VI) capable of being folded into membrane-spanning α-helices, as originally suggested for the ADP/ATP translocase [38]. Because in the ADP/ATP translocase, in the phosphate and citrate carriers and in the uncoupling protein the hydrophobicities of segments II and IV are weaker than those of

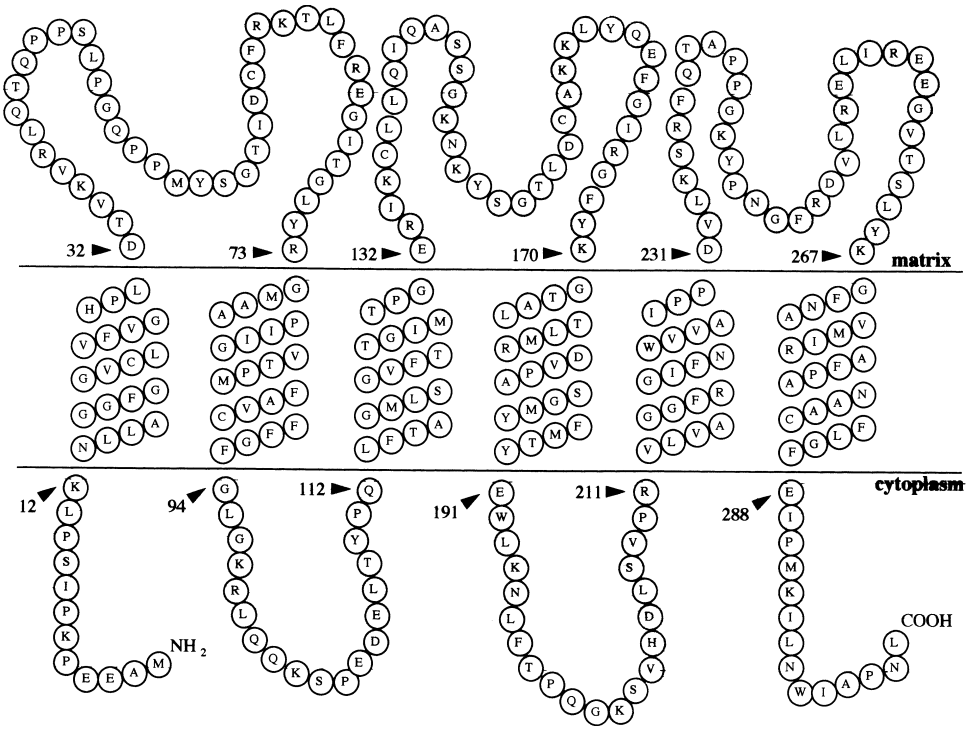

Figure 4 Proposed model for the arrangement of the CAC protein in the inner mitochondrial membrane

The model is based on the hydrophobic profile of the carrier protein. The hydrophobic segments are shown as six transmembrane α-helices (two in each repetitive domain) connected by extramembranous hydrophilic loops. Numbers refer to the locations of specific residues within the primary sequence of the CAC protein. The N- and C-terminal regions of the CAC polypeptide chain are predicted to protrude towards the cytosol because all the mitochondrial carrier proteins investigated so far exhibit a cytoplasmic exposure of their extremities [43–49].

segments I and III, alternative models have been proposed [40–42]. However, the hydrophobicity of segment II is greater in the CAC than in the other proteins. Although definitive proof will only be given by the crystal structure, many of the predictions derived from the model with six α-helices have been confirmed experimentally by investigating the accessibility of the extramembranous regions of various carriers to impermeable reagents such as peptide-specific antibodies and proteolytic enzymes [43–49]. Furthermore it should be considered that the tripartite structure strongly implies that each of the three domains in the six carrier proteins has related secondary and tertiary structures, thus making any model including differences between the three domains unlikely. On the basis of the hydrophobic profile of the CAC, its tripartite structure and its sequence similarity with the other mitochondrial carriers, a model of the proposed folding of the CAC in the inner mitochondrial membrane is shown in Figure 4. In addition to the presence of six transmembrane α-helices connected by five hydrophilic loops and to the cytoplasmic exposure of the N-and C-terminal regions, the proposed secondary structure of the CAC (Figure 4) points to another characteristic of the mitochondrial carriers, i.e. their asymmetric nature. The asymmetric orientation of the membrane-embedded CAC was previously suggested on the basis of functional studies indicating different substrate-binding sites on the inner and outer faces both of intact mitochondria [12] and reconstituted liposomes [17]. Furthermore the hydrophilic loops of the CAC (except for that connecting transmembrane α-helices II and III) have a net positive charge as observed for the other mitochondrial carriers, although carnitine and acylcarnitines are zwitterionic compounds. In a similar way to the other sequenced mitochondrial carriers, the CAC displays a net positive charge also in the intramembranous portion of the protein. In the CAC a positive charge is present in the transmembrane α-helices I, IV, V and VI. The only intramembranous negatively charged residue of the CAC is present in helix IV. This feature is shared only by the uncoupling protein, which has another negative residue in helix I.

The information derived both from the sequence comparison (Figure 3) and from the proposed secondary structure (Figure 4) indicates that the junctions between the hydrophobic and hydrophilic regions of the mitochondrial carrier proteins are the most highly conserved [21]. However, in contrast with a previous suggestion [41], relatively high conservation also occurs in the transmembrane α-helices. The extensive hydrophilic extramembranous loop A also displays significant conservation, which decreases in loops B and C, and is virtually absent from the shorter loops between the three repeats (see Figure 3).

Number of genes for CAC

To investigate the number of sequences related to the CAC-coding region in the rat and human genomes, Southern blot analysis of digests of rat and human DNA was performed by using the rat 824 bp cDNA fragment (generated by PCR-3) as a hybridization probe. The restriction enzymes *Eco*RI, *Hin*dIII and *Xho*I employed for this analysis were chosen on the basis of the observation that compatible restriction sites were absent within the 1230 bp cDNA encoding the CAC protein. As shown in Figure 5, both genomic DNAs digested with *Eco*RI yielded a single band; with *Hin*dIII and *Xho*I it yielded two bands. The presence of two hybridization bands after treatment with *Hin*dIII or *Xho*I can be explained by assuming a second gene, or a

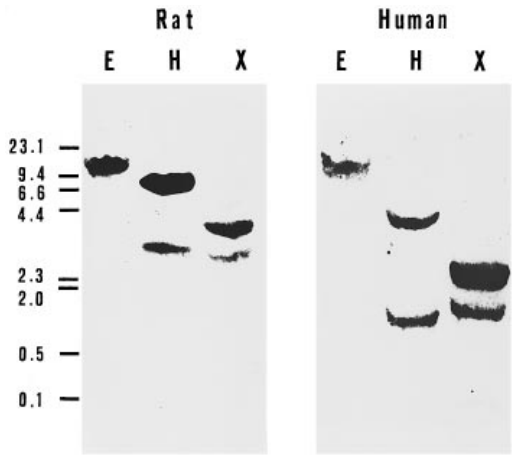

Figure 5 Hybridization of restriction digests of rat and human DNA with a probe consisting of the rat 824 bp cDNA clone generated by PCR-3

Genomic DNA from rat and man were digested with the restriction enzymes *Eco*RI, *Hin*dIII and *Xho*I, denoted by E, H and X respectively. The probe was nucleotides 406–1230 (see Figure 2 and the Experimental section). The positions of the DNA markers (in kb) are shown at the left.

pseudogene, for the CAC. In relation to this is the finding that a number of mammalian mitochondrial membrane proteins have more than one expressed gene [50–53]. Alternatively, the coding region of a single gene for the CAC might be divided by a number of introns, which contain a *Hin*dIII and an *Xho*I restriction site (and not an *Eco*RI site). This possibility is in agreement with the detection of a single band after cleavage of the rat and human genomes with *Eco*RI (Figure 5) and the existence of multiple introns in the genes for the mitochondrial carriers [30–31,52–54]. Finally, we cannot exclude the existence of other more distantly related sequences in the rat and human genomes that were not detected under the conditions of high stringency used in our experiments.

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