# Transmembrane Topology, Genes, and Biogenesis of the Mitochondrial Phosphate and Oxoglutarate Carriers

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Phosphate and oxoglutarate carriers transport phosphate and oxoglutarate across the inner membranes of mitochondria in exchange for $OH^-$ and malate, respectively. Both carriers belong to the mitochondrial carrier protein family, characterized by a tripartite structure made up of related sequences about 100 amino acids in length. The results obtained on the topology of the phosphate and oxoglutarate carriers are consistent with the six α-helix model proposed by Saraste and Walker. In both carriers the N- and C-terminal regions are exposed toward the cytosol. In addition, the oxoglutarate carrier has been shown to be a dimer by means of cross-linking studies. The bovine and human genes coding for the oxoglutarate carrier are split into eight and six exons, respectively, and five introns are found in the same position in both genes. The bovine and human phosphate carrier genes have the same organization with nine exons separated by eight introns at exactly the same positions. The phosphate carrier of mammalian mitochondria is synthesized with a cleavable presequence, in contrast to the oxoglutarate carrier and the other members of the mitochondrial carrier family. The precursor of the phosphate carrier is efficiently imported, proteolytically processed, and correctly assembled in isolated mitochondria. The presequence-deficient phosphate carrier is imported with an efficiency of about 50% as compared with the precursor of the phosphate carrier and is correctly assembled, demonstrating that the mature portion of the phosphate carrier contains sufficient information for import and assembly into mitochondria.

**KEY WORDS:** Mitochondria; carriers; sequences; transmembrane topology; genes; biogenesis.

## INTRODUCTION

The inner membranes of mitochondria contain at least 12 proteins to transport various metabolites into and out of the matrix space (Krämer and Palmieri, 1992; Walker, 1992). Of these, the phosphate carrier (PiC) and the oxoglutarate carrier (OGC) have been thoroughly studied. The PiC catalyzes the uptake of phosphate either by proton cotransport or in exchange for hydroxyl ions, and is essential for the synthesis of ATP during oxidative phosphorylation. The OGC transports 2-oxoglutarate across the mitochondrial membrane in exchange for malate or other dicarboxylic acids, and plays an important role

in several metabolic processes including gluconeogenesis from lactate, the malate-aspartate shuttle, and nitrogen metabolism. Both carriers have been purified and reconstituted into liposomes in the active form. The PiC, the OGC, the ADP/ATP carrier, and the uncoupling protein from brown adipose tissue are the only four mitochondrial carriers of known function so far sequenced. These proteins are formed by three segments of about 100 amino acids, which are homologous to one another. The clear homology between these carriers led to the development of a concept of a carrier family, which is supposed to have originated from a common gene (Aquila *et al.*, 1987; Runswick *et al.*, 1987). It is likely that also the other biochemically well characterized mitochondrial carriers fall into the same family since they have a very similar *Mr* of around 30 kDa and

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tend to copurify (Krämer and Palmieri, 1989, 1992). This review will focus on the structure, gene organization, and biogenesis of the PiC and the OGC.


## PRIMARY STRUCTURES AND STRUCTURAL RELATIONSHIP OF THE MITOCHONDRIAL CARRIER PROTEIN FAMILY

Our main contribution to the elucidation of the primary structure of the mitochondrial carrier proteins has been the cloning and sequencing of the cDNA coding for the OGC. First, several protein sequences were obtained from the protein we had purified to homogeneity from porcine and bovine heart mitochondria (Bisaccia et al., 1985; Indiveri et al., 1987). On the basis of these partial sequences, several oligonucleotides were synthesized and employed as hybridization probes in attempts to isolate clones encoding the OGC from a bovine heart cDNA library. All of these experiments, however, were unsuccessful, due to the low level of expression of the OGC. Finally, in collaboration with Dr. J. E. Walker, we adopted a new strategy based upon the polymerase chain reaction (PCR) (Runswick et al., 1990). In a first set of PCRs, degenerate oligonucleotides corresponding to the N- and C-terminal ends of available protein sequences were used as forward and reverse primers to amplify specific segments of a cDNA made from bovine heart poly($A^+$) mRNA. The products of the reaction were cloned and clones of interest were recognized with hybridization probes based upon the central regions of the available protein sequences. By this procedure, short cDNAs encoding the central part of the available protein sequences were obtained. These central sequences were then used to synthesize specific primers for further PCR experiments. From the sequences of several overlapping cDNA clones the complete cDNA sequence was obtained (Runswick et al., 1990). It was 1217 nucleotides in length and was terminated by a poly(A) tail separated by 22 nucleotides from the preceding sequence AATAAA, a typical signal for polyadenylation of RNA. This cDNA sequence encoded a protein of 314 amino acids including the initiator methionine, with a calculated Mr of 34 K. The polypeptide sequence predicted from the DNA sequence agreed with the available amino acid sequences of the purified OGC. One of these sequences corresponded to residues 5–19

of the entire protein. Therefore, the OGC has no processed import presequence.

Comparison of the protein sequence with itself revealed that the OGC has a tripartite structure made of related sequences about 100 amino acids long. Moreover, the repetitive elements of the OGC were related to those found in the ADP/ATP carrier, the PiC, and the uncoupling protein (Fig. 1), showing that the OGC is a member of the mitochondrial carrier protein family. The hydrophobic profiles and the alignment of the 12 related sequences (Runswick et al., 1990) clearly indicated that these four proteins have a similar structure with six hydrophobic domains, probably folded in the membrane as α-helices, linked by extramembranous loops. The proposed extramembranous regions, which are hydrophilic, are among the most variable parts of the proteins, and presumably they are the sites of the substrate-binding centers and the gates. In contrast, the junctions between the hydrophobic and the hydrophilic regions are the most highly conserved. A distinctive feature is the full conservation of three proline residues at the ends of helices I, III, and V, three glycines at the beginning of helices II, IV, and VI, and an acidic amino acid two residues after the prolines. The characteristic sequence features of the mitochondrial carrier family, i.e., the tripartite structure and the conserved sequence of motifs, have been used to identify other proteins of unknown function as belonging to the mitochondrial carrier family, such as MRS3 and MRS4 from *Saccharomyces cerevisiae* (Weisenberger et al., 1991), a protein from the ciliated protozoa *Oxytricha fallax* (Williams and Herrick, 1991), five members from the nematode worm *Caenorhabditis elegans* (Runswick et al., 1993), and a protein associated with Grave's disease (Zarrilli et al., 1989). This last protein (the Grave's disease carrier or GDC) was cloned by Zarrilli et al. (1989) from a human thyroid cDNA expression library with the aid of circulating autoantibodies associated with Grave's disease. We then cloned and sequenced the bovine homologue of the GDC and showed that it is expressed generally, not only in the thyroid (Fiermonte et al., 1992). Figure 1 shows the alignment of all the known sequences of bovine mitochondrial carriers including those of the GDC.

We also isolated and characterized a full-length cDNA clone encoding the precursor of the human heart mitochondrial PiC protein (Dolce et al., 1991). The entire clone was 1330 bp in length with 5' and 3'- untranslated regions of 48 and 184 bp, respectively.

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              SDQALSFLKDFLAGGVAAAISKTAVAPIERVKLLLQVQHASKQISAEKQYKGIIDCVVRIP--KEQGFLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLG
              ---------GVDRHKQFWRYFAGNLASGGAAGATSLCFVYPLDFARTRLAAD---VGKGAAQREFT-GLGNCITKIFKSDGLRGLYQGFNVSVQGIIIYRAAYFGVYDTAKGMLP-
    AAC       --------------DPKNVHIIVSWMIAQTVTAVAGLVSYPFDTVRRRMMMQSGRKGADIM--YTGTVDCWRKIAKD--EGPKAFFKGAWSNVLR-GMGGAFVLVLYDEIKKFV
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              VNPTTSEVHPTMGVKIFSAGVAACLADIITFPLDTAKVRLQIQGEGQISSTIR-YKGVLGT--ITTLAKTEGLPKLYSGLPAGIQRQISFASLRIGLYDTVQEYFSS
              ------------GKETPPTLGNRISAGLMTGGVAVLIGQPTEVVKVRLQAQ---SHLHGIKPRYT-GTYNAYRIIATTESFSTLWKGTTPNLLRNVIINCVELVTYDLMKGALV-
    UCP       -----------NNQILADDVPCHLLSAFVAGFCTTFLASPADVVKTRFINS--------LPGQYPSVP-SCAMTMLTKEGPTAFFKGFVPSFLRLASWNVIMFVCFEQLKKELSK
              SRQTVDCTT
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              AVEEQYSCDYGSGRFFILCGLGGIISCGTTHTALVPLDLVKCRMQVD--------PQKYKSIFNGFSVTL--KEDGFRGLAKGWAPTFIGYSLQGLCKFGFYEVFKVLYSN
              --------MLGEENAYLWRT-SLYLAASASASAEFFADIALAPMEAAKVRIQTQ----------PGYANTLRDAAPKMYKEEGLKAFYKGVAPLWMRQIPYTMMKFACFERTVEALYK
    PiC       -----FVVPKPRSECSKPEQLVVTFVAGYIAGVFCAIVSHPADSVVSVLNKEKG---------------SSASEVLKRLGFRGVWKGLFARIIMIGTLTALQWFIYDSVKVYFRL
              PRPPPPEMPESLKKKLGYTQ
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              MAATASPGASGMDGKPRTSPKSVKFLFGGLAGMGATVFVQPLDLVKNRMQLSGEG---AKTREYKTSFHALISIL--RAEGLRGIYTGLSAGLLRQATYTTTRLGIYTVLFER---
              ----------LTGADGTPPGFLLKAVIGMTAGATGAFVGTPAEVALIRMTADGRLPVDQRR--GYKNVF-NALFRIVQEEGVPTLWRGCIPTMARAVVVNAAQLASYSQSKQFL--
    OGC       -----------LDSGYFSDNILCHFCASMISGLVTTAASMPVDIVKTRIQNMRMID----GKPEYKNGLDVLV-KVVRYEGFFSLWKGFTPYYARLGPHTVLTFIFLEQMNKAYKR
              LFLSG
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MAAAAAALAATEPPPAMPQAAGAGGPAARRDFYWLRSFLAGGIAGCCAKTTVAPLDRVKVLLQ--------AHNHHYRHLGVFSTLRAVPKKEGYLGLYKGNGAMMIRIFPYGAIQFMAFEHYKTLITT
              --------------KLGVSGHVHRLMAGSMAGMTAVICTYPLDMVRVRLAFQ------VKGEHTYTGIIHAFKTIYAKEGGFLGFYRGLMPTILGMAPYAGVSFFTFGTLKSVGLS
    GDC       YAPTLLGRPSSDNPNVLVLKTHINLLCGGVAGAIAQTISYPFDVTRRRMQLGAVL-------PEFEKCLTMRE-TMKYVYGHHGIRKGLYRGLSLNYIRCVPSQAVAFTTYELMKQ
              FFHLN                                      * *                                             **   ***
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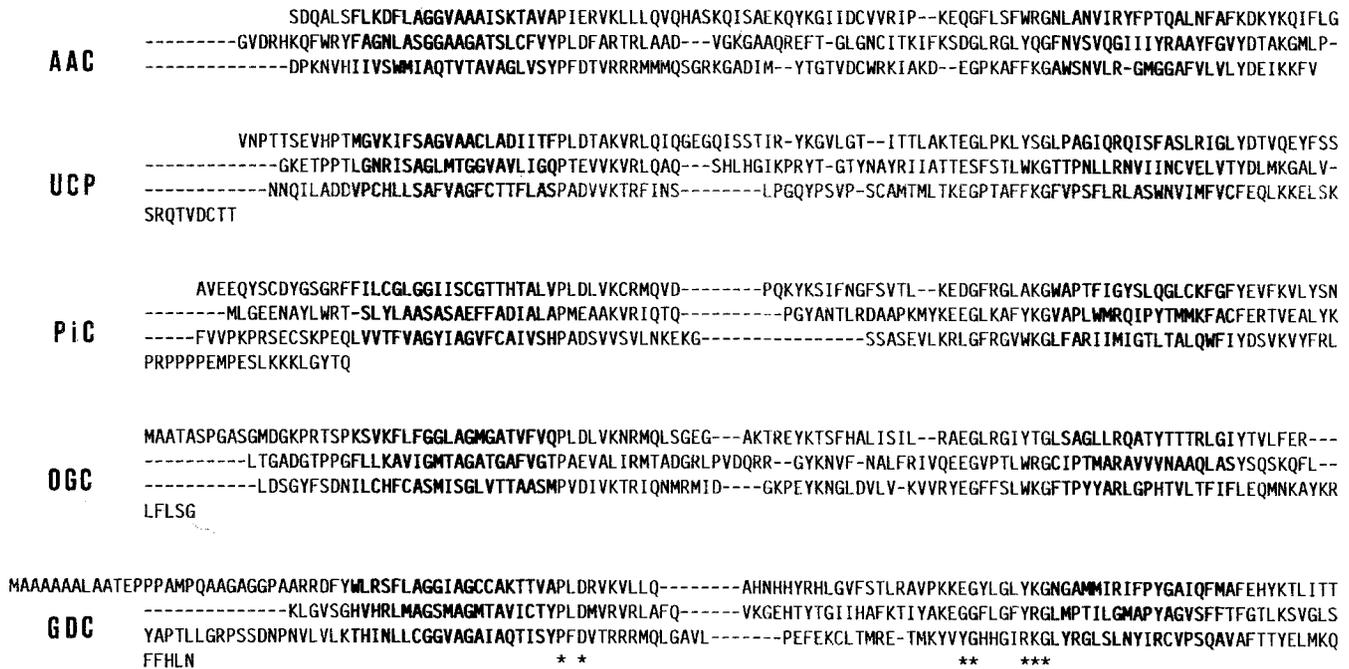
Fig. 1. Alignment of the amino acid sequences of the five known bovine mitochondrial carriers. The alignments are based upon the dot plots. The hydrophobic regions are shaded. Abbreviations: AAC, ADP/ATP carrier; UCP, uncoupling protein; PiC, phosphate carrier; OGC, oxoglutarate carrier; GDC, Grave's disease carrier.

The open reading frame encoded the mature protein consisting of 312 amino acids, preceded by a presequence of 49 amino acids. The mature human PiC sequence differs in 21 amino acids from that of the bovine heart (Runswick et al., 1987) and in 18 amino acids from that of rat liver (Ferreira et al., 1989). Out of these differences, six between man and cow and two between man and rat are nonconservatives. The PiC from man is much less homologous with that from yeast (Phelps et al., 1991) (33%), although the homology increases to 49% if only the hydrophobic regions are considered.

## SECONDARY STRUCTURE AND MEMBRANE TOPOLOGY OF PiC AND OGC

By 1982 Saraste and Walker had already proposed a model for the ADP/ATP carrier in which each of the three repeated elements consisted of two transmembrane α-helices forming a structure with six α-helices in total (I–VI). The two helices of the individual elements are linked by an extensive more polar segment (A, B, and C), whereas the three repeats are linked by somewhat shorter stretches of hydrophilic

amino acids (a' and b'). It is likely that also the other mitochondrial carriers sequenced so far share a similar arrangement in the lipid bilayer, because their hydrophobic profiles are very similar. We have investigated the transmembrane organization of the PiC (Capobianco et al., 1991; Palmieri et al., 1992a) and of the OGC (Bisaccia et al., 1993) by examining the exposure of these proteins on the cytoplasmic and matrix sides of the inner membrane to peptide-specific antibodies and proteolytic enzymes. The picture which has emerged is in agreement with the six-helix model. An important prediction of this model is that the amino- and the carboxy-extremities of the polypeptide chain are exposed to the same side of the membrane (because of the even number of the transmembrane segments). The PiC is the only mitochondrial carrier, of which the exposure of both termini on the same side of the inner mitochondrial membrane has been documented so far (Capobianco et al., 1991). In particular, we have shown that both the N- and C-terminal regions of the PiC are exposed toward the cytoplasmic side of the inner mitochondrial membrane on the basis of the following observations: (i) Both in freeze-thawed mitochondria (Capobianco et al., 1991) and in mitoplasts (Palmieri et al., 1992a and

unpublished data), in which the outer membrane is damaged or removed, the two extremities of the PiC were accessible to antibodies directed against the amino acid sequences 1–10 and 303–313 of the bovine heart PiC. (ii) Carboxypeptidase A markedly decreased the binding of anti-C-terminal antibodies (but not of anti–N-terminal antibodies) to the membrane-bound PiC (Capobianco *et al.*, 1991). (iii) Proteolysis of the membrane-bound PiC with trypsin strongly decreased the immunoreaction of the carrier with both the anti-N- and anti-C-terminal antibodies (Palmieri *et al.*, 1992a and unpublished data). A cytosolic location of the C-terminus of the PiC has also been proposed by Ferriera *et al.* (1990). In addition to the amino- and carboxy-terminal regions of the PiC, other parts of its polypeptide chain have been found to be exposed to the water face by proteolysis. In freeze-thawed mitochondria (Palmieri *et al.*, 1992a) and mitoplasts (unpublished data) the Lys-endoprotease cleaved after Lys-96, Lys-198 (or Lys-203), and Lys-288 (in loops a' and b' and in the C-terminal region). In submitochondrial particles Arg-endoprotease cleaved after Arg-140 (or Arg-152) (Capobianco *et al.*, 1991). These experiments provided evidence of the matrix exposure of loop B and of the cytoplasmic exposure of loops a' and b', and the cleavage sites are all compatible with the six-helix model.

Recently, in still unpublished studies, we employed mitoplasts and proteoliposomes, reconstituted with purified OGC, to investigate the transmembrane arrangement of the OGC polypeptide chain. Since the OGC is a rare component of the mitochondrial membrane, proteoliposomes proved to be very useful, given the difficulty of purifying the peptides generated from the OGC by the action of proteases from mitoplasts. Furthermore, in addition to "normal" proteoliposomes we used "inside-out" proteoliposomes in which the membrane was partially inverted by the freeze-thaw-sonication procedure. The latter particles are also closed vesicles and catalyze an active oxoglutarate/oxoglutarate exchange as "normal" proteoliposomes do. We found that the anti-C-terminal antibodies (specific for the amino acid sequence 303–314 of the bovine heart OGC) were bound to intact bovine heart mitoplasts, indicating that the C-terminal region of the OGC is exposed to the cytoplasmic side of the inner mitochondrial membrane. This region is located exclusively at the external side of the inner membrane, since the reactivities of the anti-C-terminal

antibodies with intact and broken mitoplasts were exactly the same. In contrast with the situation found in the mitoplasts, the anti-C-terminal antibodies did not react at all with "normal" proteoliposomes; instead they reacted strongly with "inside-out" proteoliposomes. These results indicate that in proteoliposomes the anti-C-terminal region of the OGC protrudes towards the inside. It should be noted that the apparent location of the C-terminus of the OGC at the cytoplasmic side of the mitoplasts and at the internal side of the proteoliposomes is in complete agreement with the conclusion based on functional data that the reconstituted OGC is oriented inside-out as compared to its orientation in mitochondria (Indiveri *et al.*, 1991). The accessibility of the OGC reconstituted in proteoliposomes to proteinase K was followed by protein sequencing and immunodetection of the cleavage products. With "normal" proteoliposomes, four cleavage sites were found between $V_{39}$ and $Q_{40}$, $Y_{61}$ and $K_{62}$, $F_{169}$ and $R_{170}$, and $R_{182}$ and $G_{183}$, indicating that these sites are exposed to the water phase at the external side of the liposomal membrane. With "inside-out" proteoliposomes, three cleavage sites (never obtained with "normal" proteoliposomes) were found between $A_5$ and $S_6$, $S_{22}$ and $V_{23}$, and $T_{103}$ and $V_{104}$, indicating that these sites are exposed to the internal side of the liposomal membrane. Assuming that the OGC in proteoliposomes is oriented inside-out as compared to mitochondria (cf. Indiveri *et al.*, 1991), these experiments provide evidence that loops A and B (cleavage sites after $V_{39}$, $Y_{61}$, $F_{169}$, and $R_{182}$) are exposed on the matrix side, and both termini and loop a' (cleavage sites after $A_5$, $S_{22}$, $T_{103}$ and immunoreactions with anti-C-terminal antibodies) are exposed on the cytoplasmic side of the inner mitochondrial membrane (Bisaccia *et al.*, 1993). As in the case of the PiC, the results obtained on the topology of the OGC are consistent with the six $\alpha$-helix model. A possible arrangement of the polypeptide chain of the OGC in the inner mitochondrial membrane based on our experimental results is shown in Fig. 2.

## DIMERIC STRUCTURE OF THE OGC

In order to investigate whether the OGC exists as a dimer, we used reagents which catalyze the formation of disulfide bridges. Upon treatment with $Cu^{2+}$-phenanthroline in the presence of 30% acetone
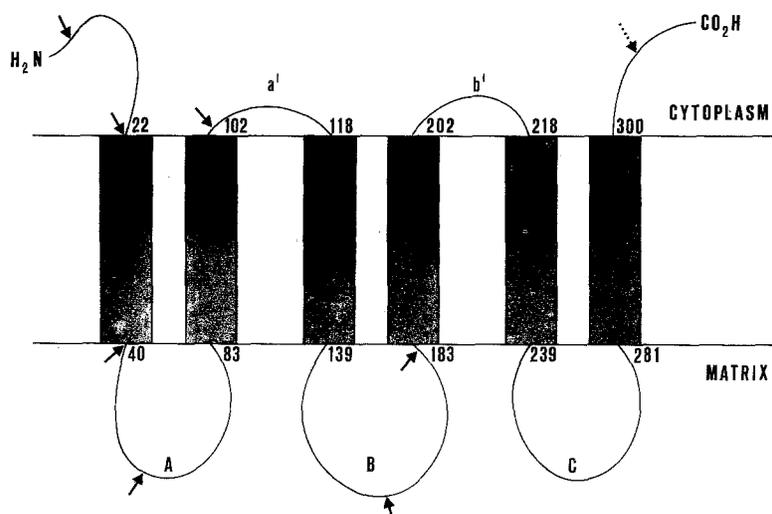
Fig. 2. Model for the transmembrane arrangement of the oxoglutarate carrier from bovine heart mitochondria. The N- and C-terminal regions of the polypeptide chain are located on the external cytoplasmic surface. Six $\alpha$-helices cross the membrane. The loop A containing the cleavage sites $V_{39}-Q_{40}$, $Y_{61}-K_{62}$ and the loop B containing the cleavage sites $F_{169}-R_{170}$, $R_{182}-G_{183}$ protrude into the matrix. The loop a' containing the cleavage site $T_{103}-V_{104}$ is exposed towards the cytosol.

or propanol, the OGC isolated from bovine heart mitochondria dimerized to a protein band with an apparent $Mr$ of 66 kDa, which was immunodetected by antibodies raised against the purified OGC. About 70% of isolated OGC was cross-linked within 10 min (Palmieri et al., 1992a, b). Several lines of evidence supported the conclusion that disulfide bridge(s) are formed between the two subunits of the preexisting OGC dimer. There are only three cysteines in the OGC polypeptide chain: Cys 184, Cys 221, and Cys 224. It has been suggested that two S–S bridges are formed at the level of the Cys 221 and Cys 224 of the two monomers (Palmieri et al., 1992b). Two other mitochondrial carriers, namely the ADP/ATP carrier and the uncoupling protein, have been shown to be dimers by means of cross-linking and ultracentrifugation studies (Klingenberg, 1981). The putative dimeric structure of the mitochondrial carriers obviously has important consequences on the mechanism(s) by which they function. Furthermore, both prokaryotic and eukaryotic membrane carriers, with very few exceptions, have in common 12 transmembrane helices. It is obvious that the mitochondrial carriers may represent a "subfamily" within the larger kingdom of carrier proteins in general if we assume that they function as dimers.

## STRUCTURE OF THE HUMAN AND BOVINE GENES FOR OGC AND PiC

Since two bovine and three human genes have been detected for the ADP/ATP carrier (Walker et al., 1987; Battini et al., 1987; Necklemann et al., 1987; Houldsworth and Attardi, 1988; Cozens et al., 1989), we investigated the possibility that more than one gene exists also for the OGC by using the bovine cDNA as a hybridization probe. By Southern blot analysis of digests of bovine and human DNA, single hybridizing bands were detected. These experiments, and also PCR's performed on bovine and human genomic DNA templates, suggested that these two genomes contain a single gene for the OGC (Runswick et al., 1990). On the basis of this information we determined the sequences of the bovine and human OGC genes (Iacobazzi et al., 1992) from overlapping genomic clones generated by PCR using primers and probes based upon the bovine cDNA. The genes are spread over 2.5 and 2.3 kb of human and bovine DNA, respectively (Fig. 3). The human gene is split into eight exons, and the bovine one into six exons, since introns C and D are present only in the former. The five introns in common interrupt the coding sequences at exactly the same position in the two

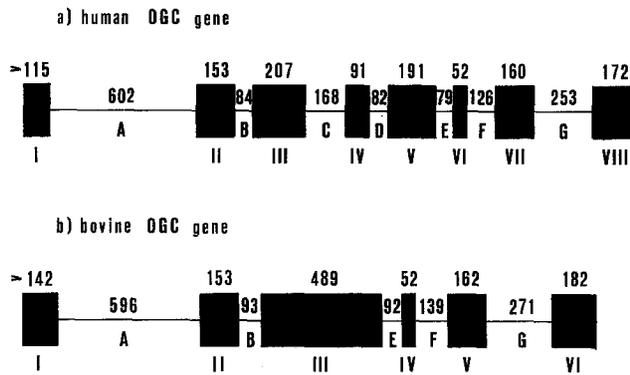a) human OGC gene



b) bovine OGC gene



Fig. 3. Structure of the human and bovine OGC genes. In each gene, the exons and the introns are shown as black boxes and solid lines, respectively.

genes. Furthermore, with one exception, all the introns in both genes have the canonical dinucleotides GT and AG at their 5' and 3' sites, respectively. The exception is the 5' splice site of the human intron G, where the dinucleotide GC is present instead of GT. The human and bovine genes for the oxoglutarate carrier are highly conserved. If introns C and D, which are both absent from the bovine gene, are ignored, they are 73% identical, and the coding sequences differ in only 70 out of 942 nucleotides. In Fig. 4 the amino acid sequences of the human and the bovine OGC are compared. A partial sequence of the rat OGC is also shown for comparison. It is clear that the homology between the OGC from these three species is very high. The human and bovine OGCs differ only in 11 amino acids. The differences are all located in the proposed hydrophilic extramembranous segments. Furthermore, in common with the bovine protein, the human OGC has no processed N-terminal sequence to target it into mitochondria.

For the PiC too, only a single gene has been detected in the bovine genome (Runswick *et al.*,

1987). We have now determined the sequences of the human and bovine PiC genes. The former was obtained by screening a human genomic DNA library in λ 2001 with two hybridization probes (the Eco RI–Eco RI fragment corresponding to nucleotides 1–562 and Eco RI–Hind III fragment corresponding to nucleotides 612–1114) derived from the human heart PiC cDNA (Dolce *et al.*, 1991) and by sequencing a clone of about 14 kb. The latter was obtained from overlapping genomic clones generated by PCR's, as previously performed for the OGC genes. The human and bovine PiC genes are 7969 and 6161 nucleotides in length, respectively. The human PiC gene contains nine exons separated by eight introns. The bovine gene has the same organization as the human one, except that the introns are generally smaller; the eight introns are found in the same position in both genes and have the canonical dinucleotides GT and AG at their 5' and 3' sites, respectively. It is interesting to note that the exon III and IV of both genes are highly homologous and they both correspond to the N-terminal region of the mature PiC. The human and bovine exons III are identical. The human exons III and IV differ in 44 out of 125 nucleotides, and the bovine ones in 41 out of 125. Furthermore, we found four human heart cDNA clones, one full length containing only exon IV and three containing only exon III. We therefore propose that in the case of the PiC gene an alternative splicing is operating, resulting in the formation of different transcripts containing either exon III or exon IV.

When considering the location of the introns of the PiC and OGC genes with respect to the six transmembrane α-helices model, it appears that there is a tendency for the introns to interrupt the coding sequences in or near to the extramembrane loops. This finding, already observed in the genes for the uncoupling protein and for the ADP/ATP carrier (Kozak *et al.*, 1988; Cozens *et al.*, 1989), presumably reflects the course of evolution of the carrier genes.

Bovine  MAATASPGASGMDGKPRTSPKSVKFLFGGLAGMGATVFVQPLDLVKNRMQLSGEGAKTREYKTSFHALISILRAEGLRGIYTGLSAGLLRQATYTTTRLGIYTVL
Human   MAATASAGAGGMDGKPRTSPKSVKFLFGGLAGMGATVFVQPLDLVKDRMQLSGEGAKTREYKTSFHALTSILKAEGLRGIYTGLSAGLLRQATYTTTRLGIYTVL
Rat     ...........................................................................................................

Bovine  FERLTGADGTPPGFLLKAVIGMTAGATGAFVGTPAEVALIRMTADGRLPVDQRRGYKNVFNALFRIVQEEGVPTLWRGCIPTMARAVVVNAAQLASYSQSKQFLL
Human   FERLTGADGTPPGFLLKAVIGMTAGATGAFVGTPAEVALIRMTADGRLPADQRRGYKNVFNALIRITREEGVLTLWRGCIPTMARAVVVNAAQLASYSQSKQFLL
Rat     .........................................................................................................LL

Bovine  DSGYFSDNILCHFCASMISGLVTTAASMPVDIVKTRIQNMRMIDGKPEYKNGLDVLVKVVRYEGFFSLWKGFTPYYARLGPHTVLTFIFLEQMNKAYKRLFLSG
Human   DSGYFSDNILCHFCASMISGLVTTAASMPVDIAKTRIQNMRMIDGKPEYKNGLDVLFKVVRYEGFFSLWKGFTPYYARLGPHTVLTFIFLEQMNKAYKRLFLSG
Rat     DSGYFSDNFLCHFCASMISGLVTTAASMPVYIVKTRIQNMRMIDGKPEYKNGLDVLLKVVRYEGFFSLWKGFTPYYARLGPHTVLTFIFLEQMNKAYKRLFLSG

Fig. 4. Amino acid sequences of the OGC from man, cow, and rat.

This course includes two tandem gene duplications of an ancestral gene coding the 100 amino acids repeat, and the 100 amino acids repeat itself may have arisen by an earlier duplication of a single hydrophobic $\alpha$-helix.

## BIOGENESIS OF THE PiC

Mitochondrial metabolite carriers are products of nuclear genes and have therefore to be imported into the mitochondrial inner membrane. The ADP/ATP carrier, the uncoupling protein, the OGC, and the yeast PiC are synthesized without a cleavable presequence (Arends and Sebald, 1984; Adrian *et al.*, 1986; Bouillaud *et al.*, 1986; Runswick *et al.*, 1990; Phelps *et al.*, 1991), whereas the mature PiC protein in cow, human, and rat is preceded by a presequence of 49 (Runswick *et al.*, 1987; Dolce *et al.*, 1991) or 44 (Ferreira *et al.*, 1989) amino acids. The presequence of the PiC, in common with other mitochondrial import sequences, has a net positive charge, but at variance with them no arginine at position -1 or -2. In order to investigate the mechanism by which the PiC is imported into mitochondria, we expressed the bovine PiC precursor in vitro and studied the characteristics of its import pathway (Zara *et al.*, 1991). The import of PiC into rat liver and bovine heart mitochondria required cytosolic factors and external ATP. The import of the PiC also required the membrane potential, $\Delta\Psi$, across the inner membrane (see also Pratt *et al.*, 1991) and occurred at membrane contact sites between outer and inner membranes. The imported protein was processed to a form with an apparent $M_r$ of 33 kDa (see also Pratt *et al.*, 1991), which gave the correct amino-terminal sequence of the mature PiC, demonstrating that the entire 49-amino-acid-residue presequence was removed. Finally, PiC was correctly assembled into the functional form, since the in vitro imported PiC showed a unique property of the PiC purified from heart mitochondria, i.e., binding to hydroxyapatite in the absence of cardiolipin and release in the presence of this phospholipid. We do not yet know if the PiC is completely translocated to the matrix compartment and retranslocated to the inner membrane or if the protein diffuses laterally from translocation contact sites to its functional location in the inner membrane.

In order to understand the role of the cleavable presequence in the import of mammalian PiC, we constructed two new plasmids coding for the mature PiC (mPiC) that lacked the entire 49-residue pre-
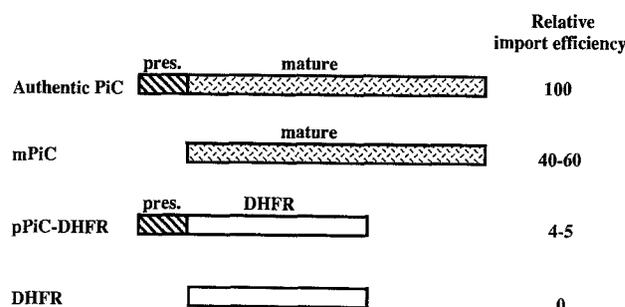


**Fig. 5.** Import efficiencies of presequence and mature part of PiC. The import efficiencies of the proteins indicated in the figure were calculated by determining the amount of protein imported to a location protected against high concentrations of proteinase K in a membrane potential-dependent manner. The import efficiency of authentic PiC was set to 100. Abbreviations: pres, presequence; DHFR, dihydrofolate reductase.

sequence and for the hybrid protein between the presequence of bovine heart PiC and the mouse DHFR (pPiC-DHFR), respectively (Zara *et al.*, 1992). We then studied the import of these proteins after their transcription and translation in vitro (Zara *et al.*, 1992). As shown for the precursor, the in vitro expressed mPiC was also imported into mitochondria, into a protease-protected location in a membrane potential-dependent process. The mPiC was imported with an efficiency of about 50% of that of authentic presequence-carrying PiC (Fig. 5). This indicates that the mature portion of PiC contains sufficient information for import, and that the presequence has a stimulatory effect but is not indispensable for the import of mammalian PiC. The import of the fusion protein into mitochondria occurred with very low efficiency (Fig. 5), in contrast to other fusion proteins between mitochondrial presequences and DHFR that were efficiently imported. This suggests that the PiC presequence alone is only a weak signal sequence. Pratt *et al.* (1991) used a truncated form of PiC that lacked most of the presequence, but contained several positive charges in front of the mature part. This mutant PiC was only very poorly imported. It is likely that the cluster of positive charges at the amino terminus of the truncated PiC is not compatible with transport into mitochondria.

What is the role of the presequence in the import of mammalian PiC? First, the presequence has an important role in determining the import specificity of the authentic PiC for mammalian mitochondria. Thus, the presequence-deficient form of mammalian PiC was imported into *Neurospora crassa* and *S. cerevisiae* mitochondria with high efficiency, whereas the

authentic presequence-carrying PiC was imported very inefficiently (Zara *et al.*, 1992). Second, the presequence enhances the translocation of PiC into the inner membrane. In an attempt to find an explanation for this effect, we studied the import of authentic PiC and mPiC into mitochondria at different concentrations of CCCP. It has been shown that intermediate concentrations of CCCP lead to a reduction of $\Delta\Psi$ and thereby differentially affect protein import (Martin *et al.*, 1991). The import of mPiC was completely blocked at $2\,\mu M$ CCCP, whereas the presequence-carrying PiC was still imported at much higher concentrations of CCCP (Zara *et al.*, 1992). This indicates that the presence of the positively charged presequence in authentic PiC allows the import of the protein at a reduced membrane potential, thus enhancing the $\Delta\Psi$-dependent transport into the inner membrane by an electrophoretic effect.

Recently we analyzed the submitochondrial location and biogenesis pathway of the PiC of *S. cerevisiae* mitochondria (Dietmeier *et al.*, 1993), which has been the object of a controversial debate in recent years. On one hand, due to primary sequence homology and reconstitution into liposomes, it was assigned to be a member of the mitochondrial metabolite carrier family and to function as phosphate carrier (Guérin *et al.*, 1990; Phelps *et al.*, 1991). On the other hand, an elegant series of experiments (Pain *et al.*, 1990; Murakami *et al.*, 1990) indicated that the protein functioned as receptor for mitochondrial precursors on the outer mitochondrial surface. By subfractionation of mitochondria we found that the yeast PiC is localized in the inner membrane (Dietmeier *et al.*, 1993). Import of the PiC precursor required a membrane potential across the inner membrane, supporting its localization in the inner membrane. This makes it unlikely that PiC functions as import receptor. Furthermore, we found that the yeast PiC precursor uses the special receptor MOM72 as its main receptor, while the "master receptor" MOM19 is required for only a minor, yet significant, portion of import (Dietmeier *et al.*, 1993). This receptor dependence has been previously observed only for ADP/ATP carrier (Steger *et al.*, 1990) and may be characteristic for all the members of the inner membrane carrier family.

## CONCLUSIONS

Undoubtedly definite progress has been made in the field of mitochondrial metabolite transport

systems (i) in elucidating an increasing number of primary structures, (ii) in envisaging a secondary structure of mitochondrial carriers, (iii) in purifying and reconstituting all the major carrier proteins (see Palmieri *et al.*, this volume) and (iv) in elucidating the kinetic mechanism of a considerable number of mitochondrial carriers (see Palmieri *et al.*, this volume). However, we still have no idea how a particular solute is translocated from one side of the membrane to the other, and we do not see a clear strategy for achieving this aim in the near future, unless the 3D-structure of a carrier protein becomes accessible. At present the most promising approach to correlating specific functions with defined structural properties of particular carrier proteins is the genetic approach, using specifically constructed mutants. In this respect, it is noteworthy that the ADP/ATP carrier and the uncoupling protein have been expressed in yeast (Klingenberg *et al.*, 1992; Klaus *et al.*, 1991; Murdza-Inglis *et al.*, 1991), and, recently, the OGC at a level of 15 mg/liter and the ADP/ATP carrier at somewhat lower levels have been expressed in *E. coli* (Fiermonte *et al.*, 1993). In the bacteria, the OGC accumulates as insoluble inclusion bodies. This material has been solubilized and reconstituted into liposomes. The transport properties of the expressed and reconstituted OGC are the same as those previously determined for the native OGC in mitochondria and the native carrier after purification from mitochondria.

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