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Mitochondrial substrate carriers

F. Palmieri, F. Bisaccia, V. Iacobazzi, C. Indiveri and V. Zara

Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari and CNR Unit for the Study of Mitochondria and Bioenergetics, Bari (Italy)

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Introduction

The inner mitochondrial membrane contains several transport systems for metabolites, which are necessary for oxidative phosphorylation, for the transfer of reducing equivalents and for important metabolic pathways such as fatty acid oxidation, gluconeogenesis and the urea cycle. So far nine mitochondrial carriers have been purified and functionally reconstituted into liposomes (for a review see Ref. 1). With the exception of the AGC, all the other carriers have been found to have a very similar molecular mass of 28–34 kDa. The primary structure of the ADP/ATP carrier (AAC), the phosphate carrier (P_iC), the uncoupling protein (UNC) and the oxoglutarate carrier (OGC) has been determined by amino acid analysis and/or by DNA sequencing. These proteins have a tripartite structure made of related sequences about 100 amino acids in length. Furthermore, the repetitive elements of each protein are related to those of the others, suggesting that these proteins belong to a carrier protein family evolved from a common ancestor gene. In this paper we report on several structural and functional properties of previously purified carriers, as well as on the first identification and purification of the aspartate/glutamate carrier (AGC) and the ornithine carrier (ORC).

Correspondence to: F. Palmieri, Dipartimento Farmaco-Biologico, Università di Bari, Traversa 200 Re David, 4, 70125 Bari, Italy.

Abbreviations: AAC, ADP/ATP carrier; AGC, aspartate/glutamate carrier; CAC, carnitine carrier; CiC, citrate (tricarboxylate) carrier; DiC, dicarboxylate carrier; OGC, oxoglutarate carrier; ORC, ornithine carrier; P_iC, phosphate carrier; UNC, uncoupling protein; $C_{12}E_8$, dodecyl-octaoxyethylene ether; HTP, hydroxyapatite; SDS, sodium dodecylsulphate.

Identification and purification of the AGC and the ORC

The identification of a carrier protein by purification and reconstitution is an important tool for its characterization at a molecular level. Since the AGC activity has been attributed to a protein with a considerably higher M_r [2] than that shown by all the other mitochondrial carriers purified so far, we reinvestigated the question of the identity of this important carrier protein [3]. The AGC from bovine heart mitochondria was solubilized with dodecyloctaoxyethylene ether ($C_{12}E_8$) and purified by chromatography on hydroxyapatite (HTP) and celite. As compared to the general isolation procedure for mitochondrial carriers, that used for the AGC employed a higher amount of HTP (10 g instead of 0.6 g) and an elution buffer containing 1 M ammonium acetate. Furthermore, once bound to celite the AGC was specifically eluted from the column by increasing the concentration of $C_{12}E_8$ from 0.1 to 0.3% in the elution buffer. On SDS-gel electrophoresis the purified AGC consisted of a single protein band with M_r of 31 500 (Fig. 1a, lane C_1). In a previous paper [2] the AGC was identified as a 68 kDa band. It is possible that this band represented a dimer of the carrier protein formed during the purification procedure and/or during the gel electrophoresis.

So far the ORC has only been reconstituted into liposomes from a Lubrol WX extract of rat liver mitochondria. We have now solubilized the ORC with 3% Triton X-100 from mitoplasts derived from rat liver mitochondria and purified it by chromatography on HTP, DEAE-Sephacel and celite [4]. In this procedure, the HTP eluate obtained in the presence of 2 mg/ml cardiolipin was applied to a DEAE-Sephacel column. The ORC was bound to this column and then eluted with 3% Triton X-100, 50 mM sodium phosphate and

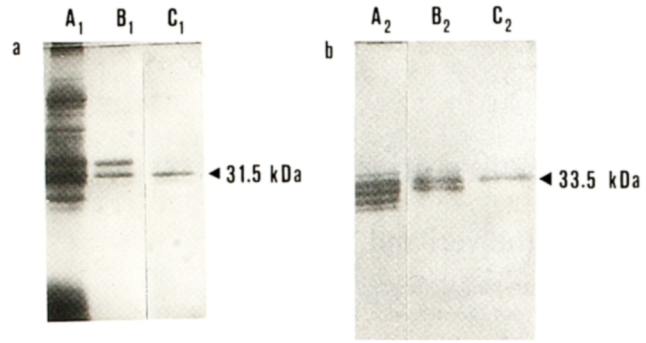

Fig. 1. Purification of (a) the aspartate/glutamate carrier from bovine heart mitochondria and (b) the ornithine carrier from rat liver mitochondria. SDS gel electrophoresis of (A_1) mitochondrial extract (5 ml); (B_1) HTP eluate (50 ml) obtained from $C_{12}E_8$ solubilized bovine heart mitochondria; (C_1) fraction (100 ml) containing pure AGC eluted from celite with 0.3% $C_{12}E_8$, 0.5 M ammonium acetate and 12.5 mM NaH_2PO_4 (pH 6.5); (A_2) HTP eluate (30 ml) obtained from Triton X-100 solubilized mitoplasts derived from rat liver mitochondria; (B_2) fraction (100 ml) eluted from DEAE-Sephacel with 3% Triton X-100, 50 mM sodium phosphate (pH 7) and 0.5 mg/ml cardiolipin; (C_2) fraction (250 ml) containing pure ORC eluted from celite with 3% Triton X-100 and 20 mM Na_2SO_4 (pH 7).

cardiolipin. The active fraction from DEAE-Sephacel was applied to a celite column, from which the ORC was specifically eluted with 3% Triton X-100 and 20 mM Na_2SO_4. On SDS-gel electrophoresis the purified ORC consisted of a single protein band with M_r of 33500 (Fig. 1b, lane C_2). When reconstituted into liposomes the ORC protein catalyzed an active mersalyl-sensitive ornithine/ornithine exchange. Citrulline was found to be the best counteranion for the transport of ornithine, followed by lysine and arginine.

Biogenesis of the P_iC

We have recently isolated and characterized a full length cDNA clone encoding the human heart mitochondrial P_iC [5]. The cDNA sequences of P_iC from bovine heart, rat liver and human heart show that the P_iC from mammals is synthesized on cytosolic polysomes as precursor. The in vitro synthesized P_iC precursors from bovine heart and rat liver were efficiently imported into isolated mitochondria and specifically processed to the mature P_iC [6,7]. Other members of the inner membrane carrier family (AAC, UNC, OGC and yeast P_iC) are synthesized in the cytosol without cleavable presequences. These proteins therefore contain the targeting information to mitochondria in their mature protein part. In order to study the role of the 49 amino-acid residue presequence of the bovine heart P_iC we synthesized a mature P_iC (mP_iC) without the amino-terminal extension and studied its transport into isolated mitochondria [8]. The presequence-deficient P_iC was imported and correctly assembled into mitochondria. The import efficiency of the mature P_iC was about 50% of that of the authentic P_iC precursor (auth. P_iC) (Fig. 2). A fusion protein between the presequence of the P_iC and the cytosolic protein dihydrofolate reductase was imported into mitochondria with very low efficiency (4–5% of that of the P_iC precursor). We therefore conclude that the mammalian P_iC contains multiple targeting signals, the more efficient one(s) present in the mature protein. Import of the mP_iC required a higher membrane potential across the inner membrane than the import of the precursor. Therefore, the presequence enhances the translocation of P_iC into the inner membrane. Furthermore, the presequence plays a role in determining the specificity of P_iC import, since the mP_iC was more efficiently imported than the P_iC precursor into mitochondria from *Saccharomyces cerevisiae* and *Neurospora crassa*.

Genes of mitochondrial carriers

The genes for the AAC and the UNC have already been characterized. We have deduced the structure of the human and bovine OGC genes from the sequences of overlapping genomic clones. These clones were generated from fragments obtained by polymerase chain reactions using pairs of primers and probes with sequences based upon the bovine cDNA. The transcribed regions of the OGC gene in man and cow spread over 2.5 and 2.3 kb of DNA, respectively [19]. As shown in Fig. 3, the human gene is split into eight exons, that of cow into six exons. The five introns in

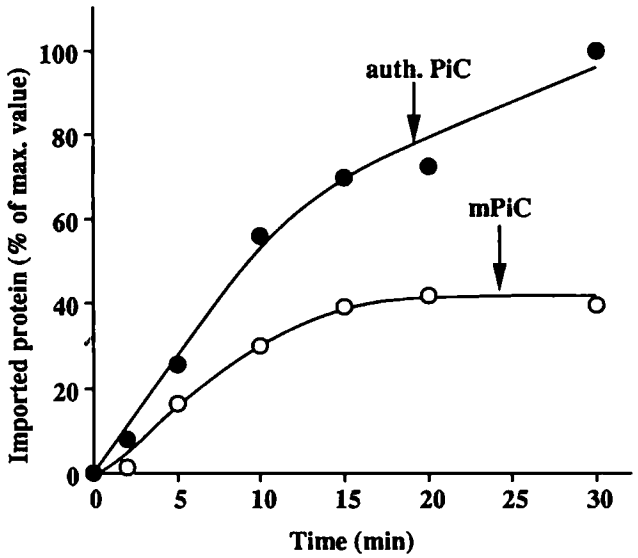

Fig. 2. Kinetics of transfer of authentic P_iC precursor and mP_iC into mitochondria. Import of authentic P_iC (●) and mP_iC (○), synthesized in reticulocyte lysates, into isolated energized rat liver mitochondria was performed at 25°C for the times indicated. After reisolation of the mitochondria the proteinase K-protected (imported) proteins were analysed by SDS polyacrylamide gel electrophoresis, fluorography and laser densitometry.

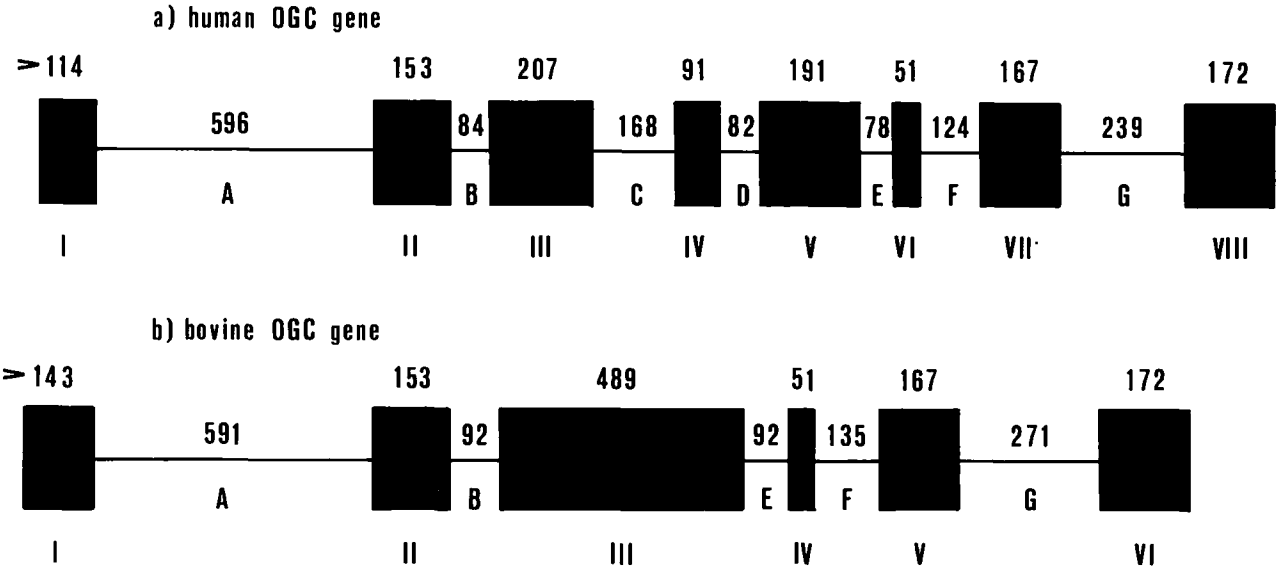

Fig. 3. Structure of the human and bovine OGC genes. In each gene, the exons and the introns are shown as black boxes and solid lines, respectively.

common interrupt the coding sequences at exactly the same positions in the two genes. The bovine and human exons exhibit 93% homology at the nucleic acid level, and 96.6% at the amino acid level. The OGC proteins in man and cow are both 314 amino acids in length and do not possess a targeting presequence [9,10]. When considering the location of the introns relative to the proposed transmembrane arrangement of the OGC, it appears that there is a tendency of the introns to interrupt the coding sequence at the boundary or outside the intramembrane hydrophobic stretches.

By applying the same methodology used for the OGC gene, we are now characterizing the human and bovine P_iC genes. These genes are considerably longer (about 6.5 kb) than the other known genes of the mitochondrial carriers, and contain at least six introns each.

In situ hybridization experiments have been performed in order to establish the chromosomal localization of the human OGC gene. A 1600 bp fragment, obtained by PCR amplification of human genomic DNA, was used as a probe. The primary amplificate was labeled with tritium and hybridized to human metaphase chromosomes. Preliminary results allowed us to map the gene on the proximal region of the long arm of a B group chromosome (number 4 or 5).

Dimeric structure of the OGC

In order to investigate whether the OGC exists as a dimer, we have used reagents which catalyze the formation of disulfide bridges. The OGC isolated from bovine heart was incubated with 0.5 mM Cu^{2+}-phenanthroline and 30% acetone or propanol for 30 min at 0°C. After denaturation of the samples at 37°C for 30 min, the degree of cross-linking was determined by SDS-gel electrophoresis. Upon treatment with Cu^{2+}-phenanthroline and the above-mentioned solvents, a protein band with an apparent M_r of 66000 appeared, which was immunodecorated by antibodies raised against the isolated OGC. About 70% of isolated OGC was cross-linked within 10 min. The solvents were probably required to cause a small perturbation of the structure and/or a local dehydration of the carrier which permit juxtapositioning of the SH groups involved in the cross-linking. Several observations give evidence for a S-S bridge-mediated cross-linking. First, the cross-linking was prevented by the SH reagents mersalyl and eosin-5-maleimide added before Cu^{2+}-phenanthroline; second, the cross-linking was not reversed by the same SH reagents added after Cu^{2+}-phenanthroline; and third, the S-S reducing reagent dithioerythrol completely reconverted the cross-linked product into the monomeric OGC. The cross-linking may depend on the dimeric state of the isolated OGC or on the collision of separate OGC molecules. Since the extent of cross-linking did not change on increasing the concentration of the OGC 7-fold, we conclude that disulfide bridge(s) are formed between the two subunits of the preexisting OGC dimer. There are only three cysteines in the bovine heart OGC polypeptide chain: Cys-184, Cys-221 and Cys-224. In order to localize the cysteines involved in the disulfide-bridge,

cross-linked OGC was cleaved at pH 9 after cyanylation of the free cysteines by 2-nitro-5-thiocyanobenzoate. On cleavage of the cross-linked OGC, a fragment with an M_r slightly lower than 30000 appeared. On the other hand, a peptide of 18 kDa was present in both cross-linked and control samples. These results give evidence, although not conclusive, that two S-S bridges are formed at the level of the Cys-221 and Cys-224 of the two monomers.

Pore-like properties of the CAC after modification by SH-reagents

The purified and reconstituted carnitine carrier from rat liver mitochondria catalyzes both heterologous carnitine/acylcarnitine exchange and unidirectional carnitine transport [11,12]. The two physiological transport modes were inhibited by mercurial reagents in the submicromolar concentration range and by *N*-ethylmaleimide. We have now found that, when 1 mM $HgCl_2$ or higher concentrations (100 mM) of mersalyl and *p*-(chloromercuri)benzenesulfonate were added to reconstituted liposomes, another transport mode of the carrier was induced [13]. This mercurial-induced uniport catalyzed by the reconstituted carnitine carrier showed a significantly reduced substrate specificity, but similar kinetics as compared to the two 'physiological' transport modes. Similar results were obtained recently with the AGC and the AAC carrier [14]. As already pointed out [14], this particular activity seems, in functional terms, to reveal an intrinsic property of at least the mitochondrial carrier protein family, i.e., a built-in unspecific channel which is normally hidden by appropriate gates. The effect of consecutive additions of various reagents including *N*-ethylmaleimide, mercurials, Cu^{2+}-phenanthroline and diamide on the transport function of the CAC revealed the presence of at least two different classes of SH-groups. *N*-Ethylmaleimide blocked the carrier activity by binding to SH-groups of class II. At least one of these SH-groups could be oxidized by the reagents forming S-S bridges. Besides binding to the class II SH-groups, mercurials also react with those of class I, the modification of which led to the induction of the carrier-mediated uniport activity characterized by loss of substrate specificity.

Species transported by the citrate carrier and reaction mechanism of mitochondrial carriers

The citrate carrier, which catalyzes a 1:1 exchange of citrate for malate, has been functionally incorporated into liposomes. The reconstituted carrier has been found to be largely inactivated below pH 7.0 and above pH 8.0 [15]. In the pH range between 7 and 8 the initial rates of citrate uptake into citrate-loaded liposomes were markedly influenced by the pH at low concentrations of citrate. In order to investigate whether the observed pH effect on citrate uptake was caused by the availability of one of the citrate species, the kinetic parameters of citrate transport were analyzed in the absence of bivalent cations. The maximal rate of citrate uptake (V_{max}) did not vary significantly between pH 7.0 and 8.0, whereas the apparent affinity (K_M) of the carrier for total citrate increased markedly with increasing pH. Calculations of the K_M at various pH values for the different citrate species showed that the K_M of $HCit^{2-}$ was remarkably constant from pH 7.0 to pH 8.0. The K_M values for all the other citrate species varied considerably with the pH and those for H_3Cit and H_2Cit^{1-} were extremely low. The K_M values of the carrier for $malate^{2-}$ were also remarkably constant whereas those for $malate^{1-}$ varied considerably. These observations strongly indicate that $HCit^{2-}$ is the only citrate species transported by the citrate carrier in exchange for $malate^{2-}$.

The reaction mechanism of the exchange, i.e., the sequence of binding and transport steps, can be investigated by two-reactant initial-velocity studies (two-substrate analysis) varying both the internal and the external substrate concentrations within the same experiment. When the kinetic data were plotted according to Lineweaver-Burk plots, a pattern of straight lines was obtained intersecting at a common point on the abscissa. This intersecting pattern, opposed by the parallel pattern of ping-pong reactions, is indicative of a sequential type of mechanism, which is observed whenever the two substrate molecules bind before catalysis occurs. Thus, the oxoglutarate carrier had to form a ternary complex with the two counter-substrates prior to translocation. Further observations are in line with this conclusion. First, the concentration-independent K_M values, obtained by extrapolation from secondary plots of the slopes and the ordinate intercepts of the primary curves vs. the reciprocal concentration of the so-called 'non-varied' substrate, agreed well with the apparent K_M values at finite substrate concentrations. Second, the K_i values, representative for substrate interaction with the unloaded carrier, more or less coincided with the corresponding K_M values. Thus, the affinity is almost identical whether binding occurs to the free or to the single-substrate occupied carrier. These results are in agreement with a special case of a sequential mechanism, i.e., a rapid-equilibrium random mechanism with fast and independent binding of the two substrate molecules. It has been shown that the reconstituted AGC [16] and OGC [17] function according to a sequential type of mechanism and we have evidence that also in the case of the purified DiC [18] a sequential mechanism is operating. Thus, four mitochondrial carriers, which catalyze strictly coupled antiport reactions, have been proven to function according to this type of mechanism.

Acknowledgements

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