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Mitochondrial metabolite transporters

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1. Introduction

The mitochondrial carriers are intrinsic proteins of the inner membranes of mitochondria that catalyze the transport of metabolites across the membrane and are necessary not only for oxidative phosphorylation but also for all the metabolic pathways, whose enzymes are partitioned between the intra- and the extramitochondrial space. So far, ten mitochondrial carriers (seven of which in our laboratory) have been purified and functionally reconstituted into liposomes (for reviews see [1,2]). These transporters are the uncoupling protein from brown fat and the ADP/ATP, phosphate, oxoglutarate, citrate, dicarboxylate, pyruvate, carnitine, aspartate/glutamate and ornithine carriers. The primary structure of the first five of these biochemically characterized transporters has been determined by amino acid analysis or DNA sequencing (for a review see [1]). These proteins are made up of three related sequences about 100 amino acids in length. Each repetitive element contains two hydrophobic stretches separated by an extensive hydrophilic region. Furthermore, the repeats of each protein are related to those present in the others. The tripartite structure, the presence of the two hydrophobic regions in each domain and the three-fold repetition of a particular sequence motif show that these five mitochondrial carriers belong to a single carrier protein family, evolved from a common ancestor by two-tandem gene duplication. The characteristic sequence features of the above-mentioned mitochondrial carriers have also been found in a number of other proteins of known sequence but of unknown function, which are therefore included in the same protein family (for a review see [1]). By examination of the transmembrane topography of the phosphate carrier, the oxoglutarate carrier and the uncoupling protein, it has been shown that the N-terminal and the C-terminal regions of these carriers protrude toward the cytosol and their polypeptide chain spans the membrane an even number of times in agreement with the six-helix model proposed for the folding of the ADP/ATP carrier (for a review see [1]). In this paper we report the results of our recent studies on several structural and functional aspects of five previously purified mitochondrial carriers.

2. cDNA cloning and primary structure of the carnitine carrier

Protein sequencing of the purified carnitine carrier (CAC) prepared according to Indiveri et al. [3] has failed to reveal an N-terminal sequence, and therefore it appears that the α-amino group of the protein is modified. On the basis of partial internal protein sequences, mixtures of oligonucleotides were used as primers and probes in attempts to isolate clones encoding the carrier from a rat liver cDNA library. Overlapping cDNA clones have been obtained that encode the entire sequence of a protein (301 amino acids long) belonging to the mitochondrial carrier family. As with other members of the family, the protein sequence contains three tandem repeats of related sequences. Furthermore, these repeats are related to those found in the ADP/ATP, phosphate, oxoglutarate and citrate carriers, and in the uncoupling protein. These results demonstrate that the CAC is a member of the mitochondrial carrier family. This idea was questioned in the past, because kinetic data, performed in liposomes reconstituted with the purified CAC, had shown that this carrier follows a ping-pong mechanism [4] at variance with all the other

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reconstituted mitochondrial carriers kinetically characterized so far, that have all been found to function according to a simultaneous (sequential) type of mechanism (for Refs. see Refs. [1] and [2]).

3. Isoforms A and B of the phosphate carrier are tissue-specific and functionally different

A single gene for the phosphate carrier (PiC) has been found in human and bovine genomes [5]. This gene has been localized to human chromosome 12q23 by fluorescence in situ hybridisation using a clone of about 15 kb encompassing the entire PiC gene [6]. The most striking feature, uncovered by the comparison of the human and bovine genomic sequences with the corresponding cDNAs of the PiC, was that both genes contain evidence of alternative splicing [5]. Thus, in both genes two exons, named III A and III B, are closely related and they appear to be alternatively spliced. Evidence that an alternative splicing of the PiC gene indeed occurs in man was obtained by characterization of cDNA clones from a human heart cDNA library. Thus, we found two cDNA containing only exon IIIA and one cDNA containing only exon IIIB [5,7]. The two alternatively spliced isoforms (A and B) differ in 13 (man) and 11 (cow) amino acids in the N-terminal region of the mature PiC protein, precisely in the segment between amino acids 4–45, which is believed to form the first of the six transmembrane α-helices of the PiC and to emerge into the first extensive extramembrane loop. In order to investigate the tissue distribution of the two transcripts of the PiC gene in cow by Northern blot analysis, we have synthesized two probes specific for bovine exon III A and exon III B, respectively, by amplifying two stretches of these exons (that were as long and as divergent as possible) on two bovine cDNA corresponding to the mature isoforms A and B (i.e., containing exon III A and exon III B respectively). The Northern blots were performed on total RNAs derived from bovine heart, skeletal muscle, liver, kidney, thyroid and lung. Transcript B was expressed in all the tissues tested although at different levels. By contrast, transcript A was expressed very strongly only in heart and skeletal muscle. With probe A no hybridisation was observed in the case of the other tissues tested. The distribution of the PiC isoforms A and B in mitochondria of various bovine tissues was investigated by Western blot analysis using anti-peptide antibodies that were specific for isoforms A and B. The anti-A antibody immunodecorated the PiC protein in SDS lysates of mitochondria from heart, diaphragm and skeletal muscle. With this antibody immunoreactivity could not be detected in mitochondrial lysates of liver, kidney, brain and lung. In contrast, the anti-B antibody immunodecorated the PiC in mitochondrial lysates from all the tissues investigated. These results clearly demonstrate the existence of two different isoforms of the PiC both at the messenger RNA level and also at the protein level. In order to investigate whether the two isoforms of the PiC are functionally different, the two bovine cDNAs corresponding to the mature isoforms A and B were cloned in a pET21b vector and over-expressed in *Escherichia coli* cells (BL21(DE3)). The two expressed PiCs carrying a histidine tail at their C-terminus were purified onto a Ni-agarose affinity column and reconstituted into liposomes. Transport experiments in the reconstituted system showed that isoforms A and B possess the same substrate specificity and the same inhibitor sensitivity but they differ in the kinetic parameters. The maximal activity (V_{max}) of isoform B was 2–3 times higher than that of isoform A, whereas the half-saturation transport constant (K_m) for phosphate of isoform B was 3 times lower than that of isoform A. These functional differences and the different levels of expression of the two isoforms in the various tissues (e.g., the high amount of the less active isoform A in heart and skeletal muscle) probably reflect tissue specificities in metabolism and energy requirement.

4. Topology, human gene and bovine cDNA of the citrate carrier

In order to investigate the topology of the citrate (tricarboxylate) carrier (CIC) in the mitochondrial membrane, we have used antibodies against two synthetic peptides corresponding to the N- and C-terminal regions of the rat-liver mitochondrial tricarboxylate carrier. ELISA tests performed with intact and permeabilized rat-liver mitoplasts showed that both anti-N-terminal and anti-C-terminal antibodies bind only to the cytoplasmic surface of the inner membrane [8], indicating that both termini of the membrane-bound tricarboxylate carrier are exposed to the mitochondrial intermembrane space. Furthermore, tryptic digestion of intact mitoplasts markedly decreased the binding of anti-N-terminal and anti-C-terminal antibodies to the tricarboxylate carrier [8]. These results are consistent with an arrangement of the tricarboxylate carrier monomer into an even number of transmembrane segments, with the N- and C-termini protruding toward the cytosol.

Like the other mitochondrial carriers, the CIC is encoded by the nuclear DNA. We have determined the sequence of the human CIC gene from overlapping genomic clones generated by PCR using primers and probes based upon the rat cDNA [9]. The gene is 2770 nucleotides in length and contains eight exons [10]. The seven introns interrupt the coding sequence in or near to the extramembrane loops. This finding, already observed in the other known genes coding for mitochondrial carriers [5,11–13], presumably reflects the course of evolution of the carrier genes, which includes two tandem gene duplications of an ancestral gene encoding the 100 amino acid repeat, and the 100 amino acid repeat itself may have arisen by an earlier duplication of a single hydrophobic α-helix. It is worth-

while mentioning that, during our investigation on the human CIC gene, we have found two human pseudogenes related to the CIC gene [10]. The sequences of these two pseudogenes, that we determined, encompass the coding sequence of the CIC gene between nucleotides 260 and 720.

We have also cloned and sequenced the bovine cDNA of the CIC. Using primers and probes based on the human cDNA sequence, overlapping cDNA clones encoding the bovine CIC protein were isolated from total bovine liver poly(A^+) cDNA. The entire bovine cDNA is 1151 bp in length with 5′ and 3′ untranslated regions of 7 and 211 bp, respectively. The open reading frame encodes a protein consisting of 311 amino acids. Assuming that the bovine CIC precursor is cleaved at the same position as that from rat [9], the mature bovine CIC consists of 298 amino acids. This sequence differs in 15 amino acids from the mature rat CIC [9] and in 14 amino acids from the mature human CIC [10]. Out of these differences 3 between cow and rat and 4 between cow and man are non-conservatives.

5. Structure-function relationships of the oxoglutarate carrier

We have continued our studies on the oxoglutarate carrier (OGC) by investigating the following aspects:

5.1. Dimeric structure of the OGC deduced from formation of a specific intermolecular disulfide bridge

We have investigated the ability of the OGC to cross-link by using disulfide-forming reagents. We found that the isolated OGC [14,15] is cross-linked to dimers by Cu^{2+}-phenanthroline or diamide, and that this dimerization is increased by acetone or some other solvents [16]. About 75% of isolated OGC was cross-linked within 10 min and the cross-linked OGC, when re-incorporated in proteoliposomes, fully retained the oxoglutarate transport activity. In addition, we found that the cross-linking depends on the dimeric state of the carrier, i.e., the dimeric state is a prerequisite for the cross-linking, since the extent of cross-linking did not change on varying the concentration of the OGC 10-fold, and dimerization was prevented by monomerization and/or degradation of the tertiary structure of the OGC. We also showed that cross-linking of OGC is accomplished by a single disulfide bond between the cysteines 184 of the two monomers. This means that the Cys-184 residues of the two subunits are fairly close to each other in the native dimer structure. It is likely that these cysteines, localized at the edge of the fourth transmembrane segment of each monomer [17], are not exactly opposite each other at the two-fold axis and possess scarce mobility, thus explaining why only monomers are found in the SDS gels of isolated OGC. In the presence of acetone, the probability that Cys-184 of the two monomers of preexisting OGC dimers should come into juxtaposition to form an S-S bridge increases and consequently a greater formation of Cu^{2+}-phenanthroline-induced cross-linked OGC is observed. These results add further evidence to the hypothesis that the functional units of the members of the mitochondrial carrier family are formed by at least two monomers.

5.2. Cys-184 is the binding site of SH reagents

It has been known for a long time that the function of the OGC is inhibited by organic mercurials and not by maleimides [18,19]. Using the reconstituted OGC, whose activity remains constant over a long period of time, we found that maleimides also inhibit the OGC activity when the proteoliposomes were incubated with these reagents for sufficiently long incubation times. This finding has allowed us to investigate the reactivity of the SH groups of the OGC by using N-(1-pyrenyl)maleimide (PM), which irreversibly binds to the OGC and whose complex with the OGC is fluorescent. OGC contains three cysteines: Cys-184, Cys-221 and Cys-224. In order to identify the cysteine(s) of the OGC labelled by PM, the reconstituted liposomes were incubated with PM, after which the carrier protein was extracted from the vesicles and subjected to BrCN cleavage. Direct sequencing of the fluorescent BrCN cleavage peptides showed that Cys-184 is the only sulfydryl group of the OGC that is alkylated by PM without prior treatment with reducing reagents [20]. Since all the other maleimides and mercurials, tested under the same experimental conditions, effectively protected the OGC against the labelling by PM, we conclude that Cys-184 is the site of the isolated and reconstituted carrier where SH-reagents bind.

5.3. Cys-221 and Cys-224 are linked by a disulfide bridge

The labelling of only Cys-184 in the reconstituted carrier by PM suggested that the other two cysteines of the OGC (Cys-221 and Cys-224) are either linked by a disulfide bridge or have a very low reactivity. To check this hypothesis, the reconstituted OGC protein was first treated with N-phenylmaleimide (FM) (in order to protect Cys-184) and subsequently with a reducing reagent (in order to break the possible disulfide bridge between Cys-221 and Cys-224). Thereafter, the carrier protein was extracted from the vesicles and labelled with PM. In the samples treated only with FM (and not with reducing reagents) the labelling of OGC by PM was completely abolished. If however, after treatment with FM the reconstituted carrier was reduced by 2-mercaptoethanol or dithioerythritol, the subsequent addition of PM led to labelling of the protein. This labelling was due to the formation of free sulphydryl groups, because it was abolished by specific SH-reagents such as mersalyl. Moreover, when 2-mercaptoethanol or dithioerythritol was added to proteoliposomes without pre-

vious treatment with FM, the PM labelling of the carrier was much greater than the control, i.e., direct labelling by PM in the absence of reducing reagent, indicating that the addition of 2-mercaptoethanol or dithioerythritol made more PM-reacting sites available per protein molecule. Furthermore, peptide sequencing showed PM labelling of only Cys-184 without preincubation with FM and reducing reagents, PM labelling of Cys-221 and/or Cys-224 after preincubation with FM and reducing reagents, and finally PM labelling of Cys-184 and Cys-221/Cys-224 after preincubation only with reducing reagents. Similar findings were obtained by using purified and SDS-denaturated OGC, instead of purified and reconstituted OGC. All these results indicate firstly, that PM binds to Cys-184 alone when the OGC protein is in the oxidized state, and secondly, that PM also binds to at least one of the other cysteines (Cys-221 or Cys-224) when the carrier is in the reduced state. Additional evidence for the existence of a disulfide bridge between Cys-221 and Cys-224 in the purified OGC came from measurements of the number of the OGC SH groups available to 5,5′-dithiobis(2-nitrobenzoate) prior and after treatment with reducing reagents. It should be noted that the existence of the proposed disulfide bridge is consistent with the earlier prediction that Cys-221 and Cys-224 are on the same face of α-helix V of the OGC [17].

5.4. The reactivity of Cys-184 to SH reagents is enhanced by substrate binding

In subsequent experiments the effect of the substrates oxoglutarate and malate was analyzed on the binding of SH-reagents to the OGC (without pretreatment with reducing reagents), i.e., to Cys-184. Liposomes reconstituted with the purified OGC were incubated with different SH-reagents with and without the two substrates. Care was taken to keep the concentration of the sulphydryl reagents sufficiently low to obtain only partial transport inhibition. Under these conditions, the inhibition of the oxoglutarate carrier activity was increased significantly by the addition of oxoglutarate or malate during the incubation of the proteoliposomes with any of the SH reagents tested. In the case of PM, by looking at its fluorescence after binding to the OGC in the presence and absence of increasing concentrations of oxoglutarate or malate, we were able to show that the substrate-induced increase in the OGC inhibition is due to an increase in the amount of maleimide bound to the carrier [20]. Interestingly, the extent of OGC labelling by PM was dependent on the amount of substrate present outside the liposomes and not on the presence of the substrate inside the liposomes. With both substrates oxoglutarate and malate, half maximal increase in transport inhibition by SH reagents and half maximal increase in the labelling by PM were obtained at a concentration close to the half-saturation transport constant (K_m) of the reconstituted carrier for external oxoglutarate or malate [21]. It should be stressed that the enhancing effect of the substrates was specific, since other chemically related anions, which are neither substrates nor inhibitors of the OGC [19,22], had no effect. The most likely explanation for the effect of the substrates on SH-reagent binding to the OGC is that substrate binding induces a change in the tertiary structure of the carrier protein which involves the region of Cys-184. As a result of the conformational change in the carrier, the reactivity or the accessibility of Cys-184 for SH-reagents is increased. A higher reactivity of Cys-184 may be caused by a decrease in the pK of Cys-184, which may occur when the microenvironment surrounding the sulphydryl group becomes more hydrophilic. In view of the putative location of Cys-184 at the border of the membrane [17], it is also possible that this residue may become more exposed to the water phase. The alternative explanation, i.e., that the increased reactivity of Cys-184 is due to an increased accessibility of the sulphydryl group to the SH-reagents, is not consistent with the finding that the increase in the inhibition induced by the presence of the substrates of the OGC is no greater for NEM or FM as compared to the bulkier PM. It should be recalled that changes in the reactivity of cysteines or other residues, as a consequence of conformational changes induced by substrate binding, have previously been reported for other transport proteins.

5.5. Site-directed mutagenesis studies

In collaboration with Dr. J.E. Walker we succeeded in over-expressing the bovine OGC in *E. coli* and refolding it in reconstitutively active form [23]. Mutations were generated by the PCR overlap extentive method using the expression vector pKN172 containing the bovine OGC cDNA as template. The mutated plasmids were employed to transform E. coli BL21(DE3) cells. All the expressed proteins were purified and reconstituted into liposomes. At first the cysteines of the OGC were selected for mutation, since they were assumed to play a role in the transport mechanism on the basis of chemical modification studies. Each cysteine of the OGC has been mutated individually (C184S, C221S, C224S) or simultaneously (C184S/C221S, C184S/C224S, C221S/C224S, C-less). In all these mutants the affinities for oxoglutarate and malate, and the V_{max} values were not significantly different from those measured for the wild-type OGC. These data provide direct evidence that the cysteinyl residues of the OGC are not essential for transport activity. Labelling experiments with PM (see above) showed the absence of the disulfide bridge between Cys-221 and Cys-224 in the expressed proteins, as normally found for the proteins expressed in *E. coli*. With the exception of the C-less mutant, all the other mutants were inhibited by mersalyl although with a different efficiency. In particular, higher concentrations of mersalyl were required for 50% inhibition of C184S/C221S, whose only remaining cysteine is

buried deep inside the membrane. Since the sulphydryl groups are not involved in the translocation mechanism, we think that the binding of SH reagents to each of the three cysteines present in the OGC has either an obstructing effect on substrate translocation or disturbs some hydrogen bonding. It is interesting that also with the expressed OGC, as with the protein purified from mitochondria (see above), the presence of the substrates oxoglutarate and malate during the incubation of the OGC with the SH-reagents increased the extent of transport activity inhibition. Further experiments with the Cys-mutants of OGC confirmed the importance of Cys-184 in the substrate-induced enhancement of the inhibitory effect of the SH-reagents on the OGC activity. The characterization of the fully active C-less mutant of the OGC provides the groundwork for further studies in which site-directed Cys replacement in C-less oxoglutarate carrier combined with chemical labeling and fluorescence spectroscopy may be used to probe the secondary and tertiary structure of the oxoglutarate carrier.

A second group of mutations was directed at the four arginine residues located in the putative transmembrane α-helices [17]. OGC proteins containing R/L mutations at each individual arginyl residue (R90L, R98L, R190L and R288L) were unable to catalyze the oxoglutarate/oxoglutarate exchange. Substitutions of arginines with lysines at positions 98 and 180 caused a strong inhibition of oxoglutarate transport. By contrast, transport activity was substantially retained when arginines at positions 90 and 288 were individually mutated to lysines. These results indicate the primary importance of intramembrane positive charges at positions 90, 98, 180 and 288 in the translocation mechanism of anionic substrates through the OGC. Furthermore they point to a specific requirement for the arginyl residues at positions 90 and 180.

6. Substrate species and reaction mechanism of the ornithine carrier

The ornithine carrier (ORC) is the last member of the mitochondrial carrier family to have been purified so far. The purified protein from rat liver [24] reconstituted into liposomes catalyzes an ornithine/citrulline exchange, which has been characterized kinetically [25]. The ornithine carrier, recently isolated from rat kidney mitochondria by us, shows the same substrate and inhibitor specificity found for the liver carrier [24]. The ornithine/citrulline exchange is very interesting physiologically, because it is involved in the urea cycle. At physiological pH, ornithine is positively charged, whereas citrulline is neutral. We therefore addressed the question of the electric nature of the ornithine/citrulline exchange. Experiments performed in proteoliposomes with the purified and reconstituted rat liver ORC showed that the exchange between ornithine and citrulline is accompanied by translocation of H^+ in the same direction as citrulline, i.e., the exchange is electroneutral, H^+ compensated. Among the experimental evidence in favour of this conclusion we may quote the following: (1) the extent of the ornithine/citrulline exchange was not influenced by the membrane potential generated by the addition of valinomycin in the presence of K^+; (2) with an intraliposomal pH of 8, the uptake of citrulline in exchange for internal ornithine was maximal at external pH 6. Vice versa, with the same intraliposomal pH, the uptake of ornithine in exchange for internal citrulline was maximal at external pH of 8–8.5; (3) the acidification of the intraliposomal space, generated by the addition of nigericin in the presence of internal K^+, stimulated the uptake of ornithine in exchange for intraliposomal citrulline; (4) the fluorescence of the pH indicator pyranine, included inside the liposomes, was decreased by the uptake of citrulline in exchange for internal ornithine, indicating an influx of H + into the vesicles together with citrulline; and (5) vice versa, the fluorescence of pyranine was increased by the uptake of ornithine in exchange for internal citrulline, indicating an efflux of H^+ from the vesicles together with citrulline.

Further experiments showed that, in addition to the ornithine/citrulline exchange, the ORC from liver catalyzes an ornithine/H^+ exchange although with a lower efficiency. The main evidence for this conclusion is the efflux of ^{14}C-ornithine from proteoliposomes in the absence of external substrate. With an intraliposomal pH of 8, this efflux was very low at external pH 8 and was increased by decreasing the pH from 8.0 to 5.5. The use of uncouplers, nigericin and pyranine (see above) gave results consistent with the occurrence of a carrier-mediated ornithine/H^+ exchange. This exchange is probably necessary to provide mitochondrial ornithine (not in exchange with citrulline) in the cytosol for biosynthetic pathways such as the synthesis of polyamines.

By two-reactant initial-velocity studies (two-substrate analysis) varying both the internal and external substrate concentrations within the same experiment, we have shown that the reconstituted oxoglutarate, citrate and dicaboxylate carriers function according to a simultaneous (sequential) mechanism, whereas the carnitine carrier proceeds via a ping-pong mechanism. We have now studied the kinetic reaction mechanism of the ornithine/ornithine and ornithine/citrulline exchanges. We found that the affinities of the ORC for the internal and external substrates are not influenced by the concentrations of the countersubstrate, and that the ratios of K_m/V_{max} (slopes of Lineweaver-Burk plots) decrease as the countersubstrate concentration increases. On the basis of this and other observations we conclude that the ornithine carrier follows a simultaneous mechanism, which implies that one internal and one external substrate molecule form a ternary complex with the carrier before the transport event occurs. This finding can be interpreted by assuming a functional dimer composed

of two subunits (each with own binding site for ornithine or citrulline) exchanging the two substrates in a concerted step between the two compartments. When comparing the reaction mechanism of the ORC with those exhibited by the other reconstituted mitochondrial carriers investigated so far, it seems that all these translocators have a common type of kinetic mechanism, with the only exception of the carnitine carrier, which is the only one that catalyzes a unidirectional transport of substrate in addition to the carnitine/acylcarnitine exchange (for Refs. see Refs. [1] and [2]).

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