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New insights about the structural rearrangements required for substrate translocation in the bovine mitochondrial oxoglutarate carrier

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ABSTRACT

The oxoglutarate carrier (OGC) belongs to the mitochondrial carrier family and plays a key role in important metabolic pathways. Here, site-directed mutagenesis was used to conservatively replace lysine 122 by arginine, in order to investigate new structural rearrangements required for substrate translocation. K122R mutant was kinetically characterized, exhibiting a significant Vmax reduction with respect to the wild-type (WT) OGC, whereas Km value was unaffected, implying that this substitution does not interfere with 2-oxoglutarate binding site. Moreover, K122R mutant was more inhibited by several sulfhydryl reagents with respect to the WT OGC, suggesting that the reactivity of some cysteine residues towards these Cys-specific reagents is increased in this mutant. Different sulfhydryl reagents were employed in transport assays to test the effect of the cysteine modifications on single-cysteine OGC mutants named C184, C221, C224 (constructed in the WT background) and K122R/C184, K122R/C221, K122R/C224 (constructed in the K122R background). Cysteines 221 and 224 were more deeply influenced by some sulfhydryl reagents in the K122R background. Furthermore, the presence of 2-oxoglutarate significantly enhanced the degree of inhibition of K122R/C221, K122R/C224 and C224 activity by the sulfhydryl reagent 2-Aminoethyl methanethiosulfonate hydrobromide (MTSEA), suggesting that cysteines 221 and 224, together with K122, take part to structural rearrangements required for the transition from the c- to the m-state during substrate translocation.
Our results are interpreted in the light of the homology model of BtOGC, built by using as a template the X-ray structure of the bovine ADP/ATP carrier isoform 1 (AAC1).

1. Introduction

The oxoglutarate carrier (OGC) is a nuclear encoded protein located in the inner mitochondrial membrane, where it catalyses the electroneutral exchange of cytosolic malate for 2-oxoglutarate from the mitochondrial matrix [1–3]. It is involved in many important metabolic processes including the malate-aspartate and the oxoglutarate-isocitrate shuttles, the gluconeogenesis from lactate and the nitrogen metabolism [3–6]. OGC was also suggested to work in tandem with CIC (citrate carrier) in the pyruvate/isocitrate cycling pathway, that is important for the regulation of glucose-stimulated secretion of insulin in pacreatic β-cells and human male gamete [7–9]. This transporter was purified, reconstituted into liposomes in a functionally active state and kinetically characterized [10–14]. The primary OGC sequence from different mammals was determined by cDNA sequencing, confirming that it belongs to the mitochondrial carrier family (MCF) [15,16]. In addition, mitochondrial OGC was the first eukaryotic membrane protein to be expressed in *Escherichia coli* and refolded in reconstitutively active form [17]. Its expression allowed important studies such as Cys-scanning mutagenesis, spectroscopic and molecular modeling studies, aimed to understand its structure-function relationships [18–27].

All of the MCF members share the same structural features, as highlighted by the determination of the atomic structure of the bovine ADP/ATP carrier isoform 1 (AAC1) in complex with its powerful

Abbreviations: OGC, Oxoglutarate carrier; WT, wild-type; MCF, mitochondrial carrier family; CIC, citrate carrier; ADP, adenosine diphosphate; AAC1, ADP/ATP carrier isoform 1; pCMBS, p-(chloromercuri)benzenesulfonic acid; MTSEA, 2-Aminoethyl methanethiosulfonate hydrobromide; MTSET, ([2-(trimethylammonium)ethyl]methanethiosulfonate bromide); MTSES, (sodium(2-sulfonatoethyl)-methanethiosulfonate); PCR, polymerase chain reaction.

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inhibitor carboxyatractyloside, that binds this carrier from the cytosolic side in the c-conformation (also known as c-state) when the internal carrier cavity is opened towards the cytosolic side and closed on the matrix side [28]. The primary sequence of all MCF members contains three tandemly repeated homologous domains of about 100 amino acids in length [3,29]; they are folded in a six transmembrane α-helical bundle (H1–H6), connected by three short α-helices (h 12, h 34, h 56) parallel to the membrane plane and located on the matrix side. H1–H6 line a water-filled cavity that contains the substrate binding site, located at the midpoint of the membrane; it contains three sites, called contact points, engaged in the recognition and binding of a specific substrate. In the neighbourhood are located several symmetrical conserved residues, supposed to be important for transport mechanism, as shown in several studies [19,20,30,31]. In each repeated domain a characteristic amino acid signature sequence motif is present: PX[D/E]XX[K/R]X[K/R] (20–30 residues) [D/E]GXXXX[W/Y/F][K/R]G [3,29]. The first part of it (PX[D/E]XX[K/R]X[K/R]) is located at the C-terminus of the odd-numbered α-helices (H1, H3, H5). The proline residues of this sequence were proposed to play a key structural role, allowing H1, H3 and H5 kinking that triggers important conformational changes. Indeed, the complete Cys-scanning mutagenesis experiments performed on the 314 residues of the *Bos taurus* SLC25A11_OGC (BtOGC) showed that the mutation of these proline residues severely impaired 2-oxoglutarate translocation [1,19,20,27,32]. Also the calculation of PhyloP and transversal scores associated to the proline residues of all MCF members highlighted that proline residues of the sequence motif are under strong negative evolutionary selection, reflecting their crucial role in substrate-translocation mechanism [33,34].

Furthermore, the charged residues of the sequence motif are crucial for substrate translocation due to their involvement in the formation of a salt bridge network, or matrix gate, that closes the carrier cavity on the matrix side (when the carrier is in the c-state) [20,28,33]. Multiple sequence alignments studies revealed the presence of a well conserved glycine residue in H1, H3 and H5, located nine residues before the first proline residue of the signature sequence [33]. Furthermore, the presence of a conserved proline residue was evidenced in the even-numbered α-helices (H2, H4, H6), positioned ten residues after the last glycine residue belonging to the second part of the signature sequence ([D/E]GXXXX[W/Y/F][K/R]G) [33]. Mutagenesis experiments [1,19,20,27,32] as well as the calculation of PhyloP and transversal scores associated to these residues also indicated that they play a crucial role in carrier function [33,34]. The hinge function of the conserved glycine and proline residues located in odd- and even-numbered α-helices is considered essential in helical rearrangements, required for opening and closing the carrier on the matrix or cytosolic side, during substrate translocation [32,33]. Since an atomic structure of a MCF member closed on the cytosolic side, when the internal carrier cavity is opened towards the matrix (known as m-state) is still lacking, the existence of a cytosolic salt bridge network, or cytosolic gate (c-gate), has only been hypothesized. The location of residues potentially involved in the formation of a c-gate was ascertained by multiple sequence alignments, that revealed the presence of a group of charged residues proposed to constitute a new sequence motif [F/Y][D/E]XX[R/K] located at H2, H4 and H6 C-terminus [30,32–34]. Charged residues of this new sequence motif were proposed to form the so-called c-gate [30,32–34]. Systematic Cys-scanning mutagenesis studies, led on BtOGC, highlighted residues essential for transport function, but the conformational changes required for the transition from the c- to the m-state (and *vice versa*) occurring during substrate translocation remain still unknown [1,19,20,27,32].

In this work, the role of lysine in position 122 (K122) in BtOGC, spatially located close to the proposed c-gate and previously reported to be involved in substrate-induced conformational changes [22] and the role of the cysteines 184, 221 and 224 was investigated, in order to gain new information about the structural rearrangements required for substrate translocation.

Our results from transport assays are explained in the light of the homology model of BtOGC, built by using as a protein template the available X-ray structure of the bovine AAC1. Evidence is provided that the lysine in position 122 doesn't takes part to the substrate binding and that cysteines 221 and 224, located near the c-gate, are involved in substrate-induced conformational changes. Furthermore the present study suggests that cysteine 184 could interfere in the formation of a functional m-state.

2. Materials and methods

2.1. Materials

2-Oxo[1-^{14}C]glutarate was purchased from Perkin Elmer (Milan, Italy); Pipes, Triton X-114, Amberlite XAD-4, bathophenanthroline-disulfonic acid disodium salt, asolectin from soybean and egg yolk phospholipids (lecithin from eggs) from Fluka (Milan, Italy); acrylamide, *N*,*N*′-methylenebis-(acrylamide) and pyridoxal 5′-phosphate from Serva (Milan, Italy); *N*-dodecanoylsarcosine (sarkosyl), p-(chloromercuri)benzenesulfonic acid (pCMBS) and mersalyl acid from Sigma-Aldrich (Milan, Italy); Sephadex G-75 from Pharmacia (Milan, Italy); 2-Aminoethyl methanethiosulfonate hydrobromide (MTSEA), ([2-(trimethylammonium)ethyl]methanethiosulfonate bromide) (MTSET) and (sodium(2-sulfonatoethyl)-methanethiosulfonate) (MTSES) from Biotium, Inc. (Hayward, California). All other reagents were of analytical grade.

2.2. Construction of plasmids and site-directed mutagenesis

The coding region for BtOGC was amplified by polymerase chain reaction (PCR) method from bovine heart cDNA [15]. The forward and reverse oligonucleotide primers corresponded to the extremities of the coding sequence for OGC [nucleotides 48–64 and 976–992 of the OGC cDNA followed by the stop codon and with NdeI and HindIII sites at the 5′ and 3′ ends, respectively].

The WT OGC cDNA was employed as a template to replace lysine 122 by arginine. K122R cDNA was employed as a template to construct triple mutants having a single cysteine residue: K122R/C184, K122R/C221 and K122R/C224. As the WT OGC contains three native cysteines (in position 184, 221 and 224), in K122R/C184 cDNA cysteines located in position 221 and 224, in K122R/C221 cDNA cysteines 184 and 224 and in K122R/C224 cDNA cysteines 184 and 221 were replaced by serines, respectively. Further mutants, named C184, C221 and C224, each containing a single cysteine residue (184, 221 and 224, respectively), were constructed in the WT OGC background, as previously described [18,20]. All of the mutations were introduced in the WT or in the K122R OGC cDNA by the overlap extension PCR method [35], using oligonucleotides with appropriate mutations in their sequences. The PCR products were cloned into the expression vector pMW7 and transformed into *E. coli* TG1 cells. Transformants selected on LB plates containing ampicillin (100 μg/ml) were screened by direct colony PCR and by restriction digestion of the purified plasmid DNA [36]. All mutations were verified by DNA sequencing and, except for the desired base changes, all of the sequences were identical to those of the WT OGC.

2.3. Bacterial expression and purification of recombinant proteins

The recombinant proteins were overproduced as inclusion bodies in the cytosol of the host cells *E. coli* CO214(DE3), as described [37].

The inclusion bodies, purified by sucrose layer density gradient centrifugation as described [38] were first washed at 4 °C with 10 mM Tris–HCl (pH 7.0), then they were washed twice with a buffer containing 10 mM Pipes (pH 7.0), 3% (w/v) Triton X-114 and once again with 10 mM Tris–HCl (pH 7.0). The WT and OGC mutated proteins were solubilized in a buffer containing 3.3% (w/v) Sarkosyl and 5 mM Pipes (pH 7.0). Residual material was removed by centrifugation (258,000*g*