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The mitochondrial dicarboxylate and 2-oxoglutarate carriers do not transport glutathione

Lee M. Booty, Martin S. King, Chancievan Thangaratnarajah, Homa Majd, Andrew M. James, Edmund R.S. Kunji *, Michael P. Murphy *

Medical Research Council Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 0XY, UK

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ABSTRACT

Glutathione carries out vital protective roles within mitochondria, but is synthesised in the cytosol. Previous studies have suggested that the mitochondrial dicarboxylate and 2-oxoglutarate carriers were responsible for glutathione uptake. We set out to characterise the putative glutathione transport by using fused membrane vesicles of *Lactococcus lactis* overexpressing the dicarboxylate and 2-oxoglutarate carriers. Although transport of the canonical substrates could be measured readily, an excess of glutathione did not compete for substrate uptake nor could transport of glutathione be measured directly. Thus these mitochondrial carriers do not transport glutathione and the identity of the mitochondrial glutathione transporter remains unknown.

1. Introduction

Mitochondria are dynamic subcellular organelles that rely on the import of nuclear-encoded proteins and the transport of metabolites and essential cofactors across the relatively impermeable inner membrane [1]. Dysfunctional mitochondrial transport is linked to a range of human pathologies [2,3]. Glutathione (GSH) is an essential small peptide present throughout the cell that has vital protective roles within mitochondria [4,5]. The mitochondrial GSH concentration is 1–5 mM and this pool exists predominantly (95–99%) in the reduced form due to NADPH-dependent glutathione reductase, whereas the remainder is in the oxidised glutathione disulphide (GSSG) form [5–7]. GSH protects against damage by degrading peroxides, detoxifying electrophiles and by interacting with protein thiols to prevent oxidative damage and mediate redox signalling (Fig. 1A) [5]. These roles are particularly vital in mitochondria, as GSH depletion within the organelle greatly increases oxidative damage, leading to cell dysfunction and death [8]. Thus the mitochondrial GSH pool plays a key role in preventing pathologies, such as neurological disorders, ischaemia/reperfusion injury and alcoholic liver disease [4,9,10].

GSH is exclusively made in the cytosol by γ-glutamylcysteine synthetase and glutathione synthetase (Fig. 1A) [11], and therefore its transport across the mitochondrial inner membrane is required [6]. Previous studies have suggested that mitochondrial GSH transport was carried out by two members of the mitochondrial carrier family (*SLC25*), the mitochondrial dicarboxylate carrier (DIC; *SLC25A10*) and the 2-oxoglutarate carrier (OGC; *SLC25A11*) [12–19]. In mammals the mitochondrial carrier family consists of 53 members, about half of which have been functionally characterised [2]. All family members have six transmembrane helices, N and C termini that protrude into the intermembrane space (Fig. 1A) and a tripartite sequence repeat of about 100 amino acids [20]. The overall structural fold of the carriers consist of six transmembrane α-helices arranged in a threefold pseudo-symmetrical way [21–23]. There are two sets of salt bridges on the matrix and cytoplasmic side of the carriers that regulate access to a central substrate-binding site for the exchange of metabolites across the mitochondrial inner membrane [24]. The mitochondrial carrier family transports many anion metabolites, such as citrate, malate,

Abbreviations: AAC, mitochondrial ADP/ATP carrier; CiC, mitochondrial citrate carrier; DIC, mitochondrial dicarboxylate carrier; GSH, glutathione; GSSG, glutathione disulphide; NEM, *N*-ethylmaleimide; OGC, mitochondrial 2-oxoglutarate carrier; TBS, Tris-buffered saline

* Corresponding authors.

E-mail addresses: ek@mrc-mbu.cam.ac.uk (E.R.S. Kunji), mpm@mrc-mbu.cam.ac.uk (M.P. Murphy).

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glutamate, ornithine and 2-oxoglutarate, so they are plausible candidates for the import of GSH (Fig. 1B).

Early studies reported mitochondrial GSH transport in isolated rat liver mitochondria through both high affinity (K_m = 60 μM, V_{max} = 0.5 nmol min^{-1} mg protein^{-1}) and low affinity transport systems (K_m = 5.4 mM, V_{max} = 5.9 nmol min^{-1} mg protein^{-1}) [25,26] that required a proton motive force. Subsequent studies have suggested that DIC and OGC were involved in mitochondrial glutathione transport. These studies used a variety of systems, such as *Xenopus laevis* oocytes [27], isolated kidney mitochondria and mitoplasts [13,14], and reconstituted proteoliposomes [16], but the GSH transport activity of these carriers was not fully explored. Therefore, we set out to characterise in detail the GSH transport capabilities of the DIC and OGC by using the well-established *Lactococcus lactis* system for overexpression and characterisation of members of the mitochondrial carrier family [28,29].

The OGC and DIC carriers were overexpressed in the cytoplasmic membrane of *L. lactis*. Isolated membrane vesicles were fused with liposomes and loaded with substrate to measure the exchange with radiolabelled metabolites [21]. Both OGC and DIC transported their canonical substrates, 2-oxoglutarate/malate and phosphate/malate, respectively. Surprisingly however, substrate competition assays with excess GSH had no effect on the transport rate in contrast to known substrates or inhibitors. Furthermore, there was no detectable transport of [^{35}S]-GSH by these carriers. Together these data demonstrate that DIC and OGC do not transport GSH.

2. Materials and methods

2.1. Materials

All materials were from Sigma–Aldrich, unless otherwise stated. M17 media (ForMedium) contained 5% (w/v) pancreatic digest of casein, 5% (w/v) soy peptone, 5% (w/v) beef extract, 2.5% (w/v) yeast extract, 0.5% (w/v) ascorbic acid, 0.25% (w/v) magnesium sulphate, 19% (w/v) disodium-β-glycerophosphate. SM17 plates consisted of M17 broth supplemented with 0.5 M sucrose and 1.5% (w/v) agar. Radiochemicals were from American Radiolabelled Chemicals and Perkin Elmer. Primers were from Sigma–Aldrich.

2.2. Molecular cloning

Codon-optimised genes for OGC (*Homo sapiens*; Uniprot ID: Q02978), DIC (*H. sapiens*; Uniprot ID: Q9UBX3, *Saccharomyces cerevisiae*; Uniprot ID: Q06143), CiC (*H. sapiens*, Uniprot ID: P53007) and AAC (*Myceliophthora thermophila*: Uniprot ID: G2QNH0) were synthesised by GenScript. For expression in *L. lactis*, the genes were cloned into the expression vector pNZ8048 and transformed into the electrocompetent *L. lactis* strain NZ9000. Successful transformants were selected on SM17 plates containing 5 μg mL^{-1} chloramphenicol, and confirmed by DNA sequencing.

2.3. Cell growth and membrane isolation

Pre-cultures of *L. lactis* were obtained by inoculating M17 medium supplemented with 1% (w/v) glucose and 5 μg mL^{-1} chloramphenicol from glycerol stocks and incubating the cultures overnight at 30 °C with no aeration. The OD_{600} was measured and cells diluted to a starting OD_{600} of 0.1 in fresh M17 medium supplemented with 1% (w/v) glucose and 5 μg mL^{-1} chloramphenicol. Cells were grown at 30 °C with no aeration until the OD_{600} reached 0.5. The expression of the recombinant proteins was induced by addition of nisin A with a dilution of 1:10000 of spent M17 medium from the nisin A secreting *L. lactis* strain NZ9700. The cells were grown for a further 2 h at 30 °C, harvested by centrifugation (6000×*g*, 10 min, 4 °C), resuspended in Tris-buffered saline, pH 7.4 (TBS) and collected by centrifugation as before. The cells were resuspended in TBS buffer and disrupted mechanically with a cell disruptor (Constant Cell Disruption Systems) at 33000 psi. Whole cells and debris were removed by centrifugation (10800×*g*, 15 min, 4 °C), and membranes were collected by ultracentrifugation (138000×*g*, 1 h, 4 °C). Pellets were resuspended in TBS buffer to a total protein concentration of approximately 5 mg mL^{-1} and stored in liquid nitrogen.

2.4. Fusion of membrane vesicles and liposomes

Escherichia coli polar lipid extract and egg yolk phosphatidylcholine (Avanti Polar Lipids) were mixed in a weight ratio of 3:1. The lipids were resuspended in TBS buffer to a final concentration of 20 mg mL^{-1} and frozen in liquid nitrogen. For membrane fusions, 1 mg *L. lactis* membranes were mixed with 5 mg liposomes, diluted to a final volume of 900 μL with TBS, and fused by seven cycles of freezing in liquid nitrogen and thawing at room temperature before storage in liquid nitrogen. The membrane vesicle fusions were thawed, and internal substrate added to a final concentration of 5 mM. Vesicles were extruded 11 times through a 1 μm pore size polycarbonate filter, passed through a pre-equilibrated PD10 column (GE Healthcare) to remove external substrate, and collected in 1.6 mL TBS buffer.

2.5. Transport assays

Transport assays were carried out using a Hamilton MicroLab Star robot (Hamilton Robotics Ltd.). Transport of radiolabeled substrate was initiated by the addition of 100 μL TBS buffer with 1.5 μM [^{14}C]-malate (2.22 GBq mmol^{-1}) or 1.5 μM [^{35}S]-GSH (16.946 TBq mmol^{-1}) to 5 μg fused membranes in a MultiScreen$_{HTS}$-HA 96-well filter plate (pore size = 0.45 μm Millipore). The transport was stopped at 0, 10, 20, 30, 45 s, 1, 2.5, 5, 7.5, 10 and 15 min by the addition of 200 μL ice-cold TBS buffer and filtering using a vacuum manifold, followed by an additional wash step with 200 μL ice-cold TBS buffer. Levels of radioactivity in the vesicles were measured by the addition of 200 μL MicroScint-20 (Perkin Elmer) and by quantifying the amount of radioactivity with the TopCount scintillation counter (Perkin Elmer). Initial rates were determined from the linear part of the uptake curves. External compounds were added at a final concentration of 10 mM, with the exception of NEM, which was added at 1 mM.

2.6. SDS–PAGE and immunoblotting

To assess protein expression, 10 μg *L. lactis* membranes were loaded onto 12% Mini-Protean Precast Tris–Glycine gels (BioRad) and run at 120 V. Gels were stained in Imperial Protein Stain (Thermo Scientific). Immunoblotting was carried out after semi-dry transfer of SDS–PAGE gels onto PVDF membrane. Membranes were incubated in 3% milk containing 1:10000 primary rabbit anti-OGC antibody for 1 h at RT. After washing, membranes were incubated in 1:10000 goat anti-rabbit secondary antibody conjugated to horse radish peroxidase for 1 h at RT. Antibody-labelled proteins were detected using the ECL reagent Western blot detection kit (GE Healthcare), following the instructions of the manufacturer, and visualised by developing the exposed film.

2.7. N-terminal modification by PCR

Primers corresponding to N-terminal regions of mitochondrial carriers known to express in *L. lactis* were designed. Extension PCR was performed using KOD Hot Start DNA Polymerase kit (Merck Millipore) on the human DIC pUC57 plasmid from