The N- and C-termini of the tricarboxylate carrier are exposed to the cytoplasmic side of the inner mitochondrial membrane

L. Capobianco[a], F. Bisaccia[a], A. Michel[b], F.E. Sluse[c], F. Palmieri[a,*]

[a]*Department of Pharmaco-Biology, University of Bari, Via Orabona 4, 70125 Bari, Italy*
[b]*Biological Chemistry Department, University of Mons-Hainaut, Avenue Maistriau 15, B-7000 Mons, Belgium*
[c]*Laboratoire de Bioénergétique, Institut de Chimie (B-6) au Sart-Tilman, Université de Liège, B-4000 Liège, Belgium*

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Abstract Polyclonal antibodies were raised in rabbits 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, 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. 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.

Key words: Tricarboxylate carrier; N-terminus; C-terminus; Antibodies; Trypsin digestion; Mitochondria

1. Introduction

The tricarboxylate carrier is a transport protein of the inner membrane of mitochondria which plays an important role in fatty acid synthesis, gluconeogenesis and the transfer of reducing equivalents across the membrane (for review see [1]). It catalyzes an electroneutral exchange of a tricarboxylate (e.g. citrate) plus a proton for either another tricarboxylate-H^+, a dicarboxylate (e.g. malate) or phosphoenolpyruvate [2–4]. The tricarboxylate carrier has been purified and successfully reconstituted into liposomes [5,6]. In the reconstituted system it has been shown that the exchange reaction proceeds via a sequential mechanism [4]. The amino acid sequence of the tricarboxylate carrier has been deduced from rat liver cDNA sequences [7]. The mature protein contains three internal repeats of about 100 amino acids in length. Furthermore, the repetitive elements of the tricarboxylate carrier are related to those of the ADP/ATP carrier, the phosphate carrier, the uncoupling protein and the oxoglutarate carrier, showing that the tricarboxylate carrier is a member of a family of mitochondrial carrier proteins. On the basis of the hydrophobic profile of the tricarboxylate carrier, Kaplan et al. [7] proposed a secondary structure model for the arrangement of the protein in the membrane in which the polypeptide chain consists of six transmembrane α-helices connected by relatively hydrophilic loops. Apart from this proposal, the transmembrane organization of the tricarboxylate carrier remains to be determined.

2. Experimental

Mitoplasts were prepared from rat liver mitochondria, suspended in an hypotonic medium consisting of 60 mM sucrose and 0.1 mM EDTA, pH 7.2, by incubating them with digitonin (1 mg/mg protein) for 15 min at 25°C. The integrity of the resulting mitoplasts was assessed by ELISA performed with anti-F_1ATPase antibodies. In agreement with the well known matrix exposure of the F_1 part of the ATP-synthetase, we found that antibodies directed against the F_1 part of the F_1ATPase did not react with intact mitoplasts whereas they reacted strongly with mitoplasts permeabilized by osmotic shock and freeze-thawing.

ELISA were performed as described previously [8,9]. Mitoplasts (500 μg in 47 μl) in 0.25 M sucrose and 10 mM Tris-HCl, pH 7.4, were digested with 3 μg trypsin for different periods of time (0, 10, and 30 min) at 10°C. The reaction was stopped by the addition of 15 μg trypsin inhibitor. Aliquots corresponding to 100 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting [8,9].

Peptides corresponding to the N-terminal region of the rat liver tricarboxylate carrier (residues 1–15: Ala-Pro-Gly-Ser-Gly-Lys-Ala-Lys-Leu-Thr-His-Pro-Gly-Lys-Ala) and to the C-terminal sequence (residues 284–298: Tyr-Asp-Glu-Val-Val-Lys-Leu-Leu-Asn-Lys-Val-Trp-Lys-Thr-Asp) were synthesized with an Applied Biosystems model 431A peptide synthesizer using Merrifield's solid-phase Fmoc/TBTU-HOBT methodology [10]. They were purified by reverse-phase chromatography on a Vydac C_{18} silica gel column (300 Å porosity, 17 μm particle size, 25 × 250 mm) using a linear gradient in the solvent system 0.1% TFA/acetonitrile. Homogeneity of the peptides was assessed by amino acid analyses and by analytical HPLC. The peptides were found to be more than 98% pure. A tyrosine residue was substituted for Ala^{15} in the N-terminal peptide to facilitate coupling to the ovalbumin carrier. The N-terminal and C-terminal peptides were coupled to ovalbumin through the hydroxyl group of Tyr, as described previously [8,9]. Antisera against the conjugates were then raised in male New Zealand white rabbits.

3. Results

3.1. Characterization of the peptide-specific antibodies

In rabbits immunized with the appropriate peptide-ovalbumin conjugates, antibodies were raised against two synthetic peptides corresponding to the N-terminal and to the C-terminal regions of the tricarboxylate carrier (see section 2). Immunological assays by ELISA showed that the anti-N-terminal antiserum and the anti-C-terminal antiserum reacted strongly with the corresponding terminal peptides without cross-reaction. Furthermore, both anti-peptide antibodies recognized specifically the isolated tricarboxylate carrier bound to microtiter plates, and also recognized the carrier among the proteins of a lysate of rat liver mitochondria after Western blotting. In all cases, no reaction was found with the preimmune sera or with

*Corresponding author. Fax: (39) (80) 5442720.

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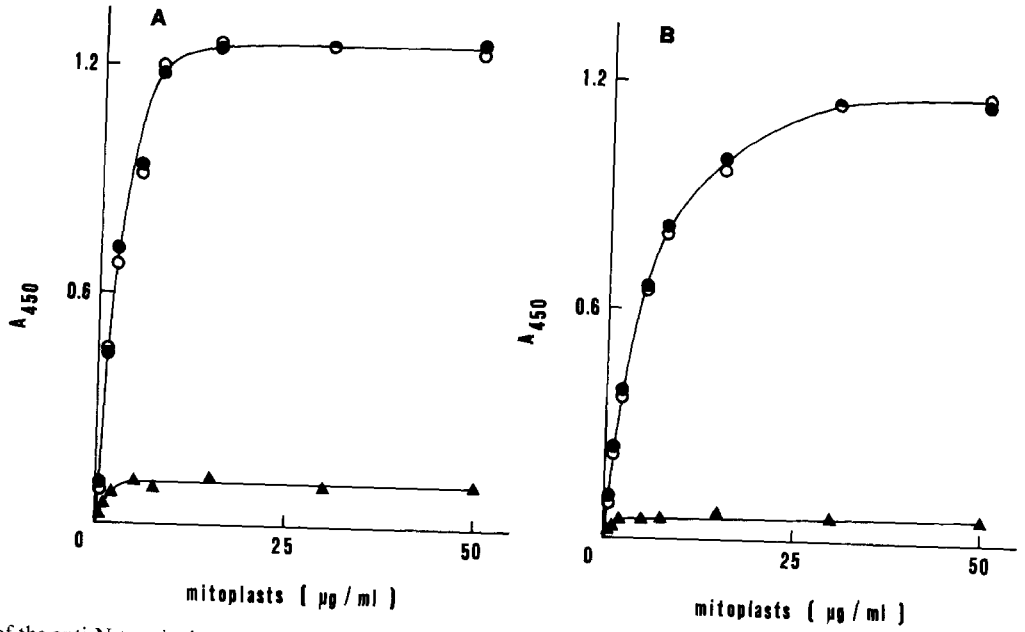

Fig. 1. Reactivity of the anti-N-terminal peptide and the anti-C-terminal peptide antisera to the membrane-bound tricarboxylate carrier in intact and permeabilized mitoplasts, assessed by ELISA. Microtiter plates were coated with the indicated amounts of intact (●) or permeabilized (○) mitoplasts. The immobilized particles were incubated with either the anti-N-terminal peptide antiserum (A) or the anti-C-terminal peptide antiserum (B) added at a dilution of $3 \cdot 10^3$. In (▲) the antisera were used in the presence of the corresponding N-terminal or C-terminal peptide at a concentration 1 µg/ml.

an antiserum directed to ovalbumin. Thus, it was possible to use these antibodies to investigate the location of the N-terminal and C-terminal regions of the tricarboxylate carrier with respect to the plane of the membrane.

3.2. *Reactivity of the anti-N-terminal and anti-C-terminal antisera with the membrane-bound tricarboxylate carrier in mitoplasts*

Investigation of the orientation of the N- and C-terminal regions in the membrane-bound rat liver tricarboxylate carrier was performed by ELISA with mitoplasts coated onto microtiter plates. The data presented in Fig. 1A,B show that the binding of the anti-N-terminal antibodies as well as the binding of the anti-C-terminal antibodies to the tricarboxylate carrier increased with the amount of coated mitoplasts. The specificity of the immunoreaction was demonstrated by carrying out similar assays in the presence of N-terminal and C-terminal peptides which drastically reduced the reaction (Fig. 1A,B). These results indicate that both the N-terminal and the C-terminal regions of the membrane-bound tricarboxylate carrier are accessible to specific antibodies from the outer side of the inner mitochondrial membrane. In order to check whether the N-terminal and the C-terminal regions of the tricarboxylate carrier are exposed, besides to the cytosolic side, also to the matrix side of the mitochondrial membrane (i.e. whether the tricarboxylate carrier is randomly inserted in the mitochondrial membrane), immunotitrations were performed with permeabilized mitoplasts, in which the matrix side of the inner membrane is also accessible to antibodies. The data presented in Fig. 1A,B show that the reactivity of the anti-N-terminal antibodies as well as of the anti-C-terminal antibodies with the tricarboxylate carrier in intact mitoplasts was not enhanced by permeabilization of the mitoplasts, indicating that the N-terminal and the C-terminal regions of the tricarboxylate carrier are located exclusively at the external side of the inner mitochondrial membrane.

It might be argued that coating the mitoplasts on the microtiter plates could disorganize the membrane structure, allowing access of anti-N-terminal and anti-C-terminal antibodies to initially non-exposed epitopes. To test this possibility, back-titration assays were performed as described by Brandolin et al. [11]. In these experiments increasing concentrations of intact mitoplasts were incubated with a fixed concentration of antisera. After centrifugation the unreacted antibodies remaining in the supernatants were back-titrated by ELISA, using microtiter plates coated with the corresponding terminal peptides. The results of back-titration ELISA are shown in Fig. 2. The reaction of the anti-N-terminal antibodies against the coated N-terminal peptide (Fig. 2A) as well as the reaction of the anti-C-terminal antibodies against the coated C-terminal peptide (Fig. 2B) markedly decreased on increasing the amount of mitoplasts, proving that 'non-coated' mitoplasts are able to bind the two terminal peptide-specific antibodies. As a control of the specificity of the immunological reactions, it was shown that when the two types of antibodies were allowed to react with the respective peptides in solution prior to being added to the suspension of mitoplasts, virtually no further reaction was detected (Fig. 2A,B).

To further investigate the transmembrane arrangement of the tricarboxylate carrier in the inner mitochondrial membrane, tryptic digestion of the carrier protein in the membrane of mitoplasts was combined with the immunological approach. In the experiments reported in Fig. 3, intact mitoplasts were incubated with trypsin for 0, 10, and 30 min (lanes 1, 2 and 3, respectively) at 10°C. After this incubation period the mitoplasts were solubilized in SDS; the proteins were separated by gel electrophoresis and immunodecorated with the anti-N-terminal antiserum in panel A and the anti-C-terminal antiserum

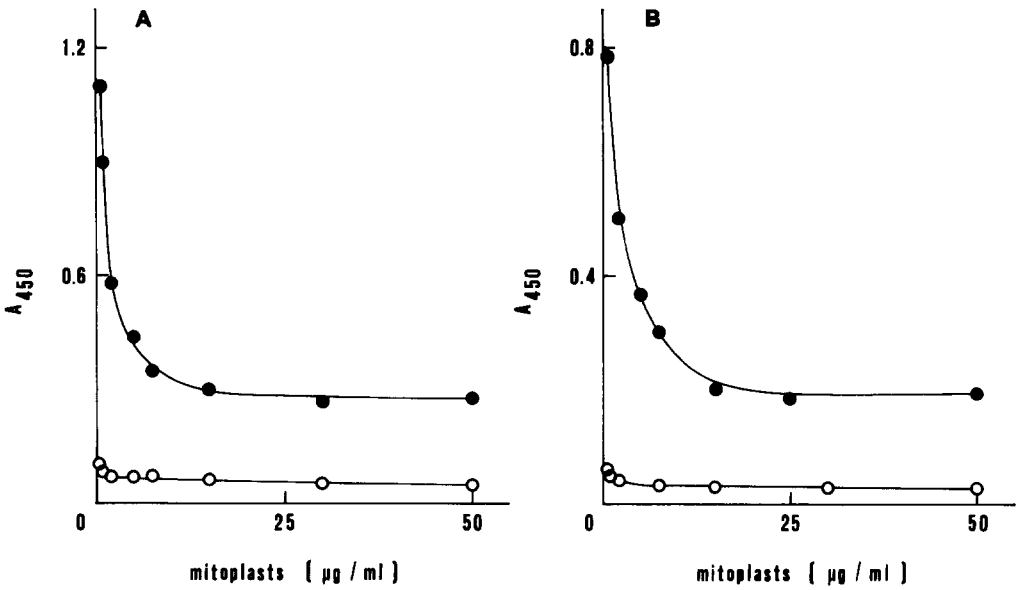

Fig. 2. Back-titration by ELISA of anti-N-terminal antibodies or anti-C-terminal antibodies after reaction with the membrane-bound tricarboxylate carrier in mitoplasts. The anti-N-terminal antiserum (A) or the anti-C-terminal antiserum (B) diluted 1,000-fold was incubated for 2 h at room temperature with mitoplasts at the indicated concentrations in the absence (●) or in the presence (○) of competing corresponding N-terminal or C-terminal peptide at 2 µg/ml. The mitoplasts were spun down by centrifugation, and unreacted antibodies present in the supernatants were assayed by ELISA, using microtiter plates coated with the N-terminal peptide (A) or the C-terminal peptide (B) at a concentration of 1 µg/ml.

in panel B. It is evident that treatment of the mitoplasts with trypsin led to a substantial decrease in the reactivity of the N-terminal and the C-terminal regions of the tricarboxylate carrier against their respective antibodies. These results are consistent with a cytosolic location of the N- and C-termini of the tricarboxylate carrier.

4. Discussion

Antibodies and proteases have been widely used to determine the sidedness of emerging segments of intrinsic membrane proteins since they are non-permeant reagents able to interact with amino acids at the surface of the membrane. In the present work we used anti-peptide antibodies and tryptic digestion to probe the orientation of the N- and C-terminal regions of the tricarboxylate carrier in the inner mitochondrial membrane. ELISA performed with intact mitoplasts coated onto microtiter plates using anti-N-terminal and anti-C-terminal antibodies demonstrated clearly the cytoplasmic exposure of the N- and C-terminal regions of the tricarboxylate carrier. These results were confirmed by back-titration ELISA experiments. As disruption of mitoplasts, which allowed the reaction of antibodies with regions of the peptide chain of the carrier exposed to the mitochondrial matrix, did not enhance the immunoreaction, it can be concluded that the tricarboxylate carrier is asymmetrically inserted in the membrane. In addition, the stepwise

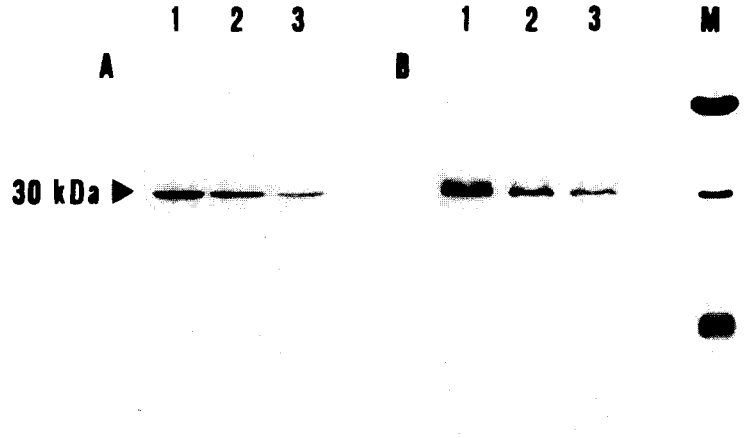

Fig. 3. Reactivity of anti-N-terminal and anti-C-terminal antisera to the tricarboxylate carrier protein treated by trypsin in mitoplasts. Intact mitoplasts were incubated with trypsin for 0, 10 and 30 min (lanes 1, 2 and 3, respectively) and immunodecorated with anti-N-terminal antiserum diluted 1,000-fold (A) and anti-C-terminal antiserum diluted 500-fold (B). Both antisera immunodecorated a band of 30 kDa corresponding to the tricarboxylate carrier. M: marker proteins (bovine serum albumin, 66,200; carbonic anhydrase, 30,000 and cytochrome *c*, 12,500).

proteolysis of the membrane-bound tricarboxylate carrier in intact mitoplasts with trypsin provided further evidence that the N- and C-termini of the protein are exposed at the cytoplasmic side of the inner mitochondrial membrane. The location of both termini of the tricarboxylate carrier on the same side of the lipid bilayer is consistent with an even number of membrane spans and with the six-helix model proposed for the folding of the ADP/ATP carrier [12].

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