Biochimica et Biophysica Acta 1848 (2015) 1393–1401

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

The mitochondrial transporter of ascorbic acid functions with high affinity in the presence of low millimolar concentrations of sodium and in the absence of calcium and magnesium

Mara Fiorani [a], Catia Azzolini [a], Liana Cerioni [a], Maddalena Scotti [a], Andrea Guidarelli [a], Caterina Ciacci [b], Orazio Cantoni [a,*]

[a] Dipartimento di Scienze Biomolecolari, Università degli Studi di Urbino "Carlo Bo", 61029 Urbino, Italy
[b] Dipartimento di Scienze della Terra, della Vita e dell'Ambiente, Università degli Studi di Urbino "Carlo Bo", 61029 Urbino, Italy

ARTICLE INFO

Article history:
Received 10 November 2014
Received in revised form 13 February 2015
Accepted 10 March 2015
Available online 15 March 2015

Keywords:
Ascorbic acid transport
Mitochondria
Ascorbic acid
Na^+-dependent ascorbic acid transporter 2

ABSTRACT

We recently reported that U937 cell mitochondria express a functional Na^+-dependent ascorbic acid (AA) transporter recognised by anti-SVCT2 antibodies. The present study confirms and extends these observations by showing that this transporter is characterised by a Km and a pH-dependence comparable with that reported for the plasma membrane SVCT2. In isolated mitochondria, Na^+ increased AA transport rate in a cooperative manner, revealed by a sigmoid curve and a Hill coefficient of 2, as also observed in intact Raw 264.7 cells (uniquely expressing SVCT2). There was however a striking difference on the Na^+ concentrations necessary to reach saturation, *i.e.*, 1 or 100 mM for the mitochondrial and plasma membrane transporters, respectively. Furthermore the mitochondrial, unlike the plasma membrane, transporter was fully active also in the absence of added Ca^{++} and/or Mg^{++}.

Taken together, the results presented in this study indicate that the U937 cell mitochondrial transporter of AA, because of its very low requirement for Na^+ and independence for Ca^{++} and Mg^{++}, displays kinetic characteristics surprisingly similar with those of the plasma membrane SVCT2.

1. Introduction

Vitamin C is a very important water soluble vitamin taken up directly by the cells as L-ascorbic acid (AA) *via* high affinity/low capacity Na^+-dependent transporters 1 and 2 (SVCT1 and 2) [1–3]. In alternative, cells may also take up the vitamin as dehydroascorbic acid (DHA) through hexose transporters [4,5], an event followed by the rapid intracellular reduction back to AA [6–8]. While this second mechanism might appear advantageous because of its high capacity, its involvement is clearly conditioned by the extracellular levels of DHA, normally in the very low micromolar range with some variation associated with the release of AA oxidizing species, as the superoxide anion [9].

Regardless of the mechanism of uptake, however, cells may accumulate large amounts of vitamin C in the cytosol and, at least in principle, in other subcellular compartments provided of adequate transport systems of the vitamin. One of such compartments is represented by the mitochondria, as these organelles actively produce reactive oxygen species and are therefore expected to benefit of the antioxidant activity of AA [10], based on direct scavenging of reactive species [11] and on other activities related to vitamin E recycling [12]. Not surprisingly, mitochondrial vitamin C has been detected in *in vivo* studies and was found to increase upon dietary supplementation of the vitamin [13–15].

The mechanism whereby the vitamin is taken up by the mitochondria has been attributed for a long time to DHA transport for at least two separate reasons: i) hexose transporters are expressed in mitochondria [15–18], despite the lack of an apparent role of glucose in mitochondrial metabolism; ii) a high capacity transport is expected to be advantageous under conditions of extensive mitochondrial formation of reactive oxygen species.

Along the same lines, the possibility of a mitochondrial AA transport has been considered unlikely because of the high affinity of SVCTs, allowing the full expression of their activity at AA concentrations remarkably lower than those detected in the cytosol of many cell types *in vivo* [19,20]. While this consideration deserves further discussion, there are two additional reasons arguing against a role of SVCTs in mitochondrial AA transport. The first one is based on the well established Na^+-dependence of these transporters [1], the activity of which does not appear compatible with the concentrations of the cation in the intracellular fluids. The second reason against the mitochondrial location (and function) of SVCT2 is based on its Ca^{++}-requirements for the expression of maximal activity [21]. Under resting conditions, there is

Abbreviations: AA, L-ascorbic acid; DHA, dehydroascorbic acid; EB, extracellular buffer; IB, intracellular buffer; MB, mitochondrial buffer; SVCT, sodium-AA co-transporter

* Corresponding author. Tel.: +39 0722 303523; fax: +39 0722 303521.
E-mail address: orazio.cantoni@uniurb.it (O. Cantoni).

http://dx.doi.org/10.1016/j.bbamem.2015.03.009
0005-2736/

about four orders of magnitude difference in Ca^{++} concentrations between the extra and intracellular compartments, with a possibility of a transient increase upon stimulation [22], however leading to Ca^{++} concentrations still remarkably lower than those found in the extracellular milieu.

The above considerations have therefore stimulated various investigations providing experimental evidence of efficient mitochondrial transport of DHA, however often using high DHA concentrations [15–18]. The available evidence is in any case in keeping with the possibility of a physio-pathological relevance of mitochondrial DHA uptake in some conditions. This mechanism has the advantage of linking DHA formation (and hence the formation of AA oxidizing species) with rapid DHA transport and reduction back to AA. In other words, AA might be rapidly accumulated in mitochondria under the same conditions in which these organelles produce the superoxide anion.

Despite these observations and logical considerations, however, we recently provided evidence for the presence of SVTC2 immunoreactivity in U937 cell mitochondria [23]. Exposure of these cells to AA was associated with the mitochondrial accumulation of even low concentrations of the vitamin. Our results were recently confirmed by another group in a different cell type [24]. These Authors, while providing solid and definitive evidence for the mitochondrial expression of a functional SVCT2, also demonstrated that because of the intracellular environment low in Na^+ and high in K^+, the mitochondrial SVCT2 functions with low affinity.

The present study, confirms and extends our previous findings [23] by showing that the U937 cell mitochondrial AA transporter is surprisingly characterised by a Km similar to that of the plasma membrane SVCT2. An additional similarity was found in the pH-dependence and positive cooperativity for Na^+ ($n_H \cong 2$). Strikingly, however, 100 fold less Na^+ was necessary to maximally support AA transport in mitochondria in comparison with the transport through the plasma membrane. A final critical difference was found for the Ca^{++}/Mg^{++} dependence, as plasma membrane SVCT2 required very high (millimolar) concentrations (normally present in extracellular fluids), in contrast with the mitochondrial transporter, fully active also in the absence of added Ca^{++}. Our results are therefore consistent with the possibility that mitochondrial SVCT2 might function with different affinities, including the very high affinity that the same transporter displays in the plasma membrane. In order to acquire this phenotype, the mitochondrial SVCT2 reduces its cation requirements for optimal activity.

2. Materials and methods

2.1. Chemicals

AA, dithiothreitol (DTT), choline-chloride, 4-Morpholineethanesulfonic acid hemisodium salt (MES) and most of the reagent-grade chemicals were purchased from Sigma-Aldrich (Milan, Italy). Perkin–Elmer Life and Analytical Sciences (Boston, MA, USA) supplied the L-[1-^{14}C]-ascorbic acid (specific activity 5.35 mCi/mmol), which was dissolved in deionized water containing 0.1 mM acetic acid and stored in multiple aliquots at −20 °C until use [25]. The antibodies against SVCT2 (sc-9926), cytochrome c (sc-7159) calnexin (sc-6465), HSP-60 (sc-13115), as well as the horseradish peroxidase-conjugated secondary antibodies (sc-2350; sc-2005) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against GLUT3 (ab53095) and lamin A (ANT0072) were from Abcam (Cambridge, UK) and Diatheva (Fano, Italy), respectively.

2.2. Cell culture and treatment conditions

U937 human myeloid leukaemia cells and Raw 264.7 murine macrophages were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Euroclone, Celbio Biotecnologie, Milan, Italy), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Euroclone). The cells were grown at 37 °C in T-75 tissue culture flasks (Corning, Corning, NY) gassed with an atmosphere of 95% air—5% CO_2. Cells (1×10^6 cells/ml) were exposed for 5 min to AA or ^{14}C-AA (specific activity 5.35 mCi/mmol) in extracellular buffer, EB (15 mM Hepes, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, pH 7.4) in the presence of 0.1 mM DTT.

A 10 mM AA stock solution was prepared in immediately before use.

Stability of AA (30 μM) in the above EB was assessed by monitoring the absorbance at 267 nm for 90 min ($\varepsilon_{267} = 14{,}600\ M^{-1}\ cm^{-1}$). In selected experiments, NaCl was replaced with choline-chloride.

2.3. Measurement of ascorbic acid content by HPLC

After treatments, the cells or mitochondria were washed twice with cold EB or IB respectively and the final pellets were extracted with ice-cold 70% (v/v) methanol/30% solution (10 mM tetrabutylammonium hydrogen sulphate, 10 mM KH_2PO_4, 0.5% methanol, pH 6.0) containing 1 mM EDTA and 10 mM DTT. After 10 min at ice bath temperature, the samples were centrifuged at 10,000 *g* for 20 min at 4 °C. Samples were filtered through a 0.22 μm filter (Millipore Corporation, Billerica, MA, USA) and either analysed immediately or frozen at −80 °C for later analysis. AA content was measured by HPLC with the UV detection wavelength set at 265 nm, as described in [26], with minor modifications. The assay involved the use of a 15 cm × 4.6 mm Discovery C-18, 5 μm column (Supelco, Bellefonte, PA, USA) equipped with a Supelguard Discovery C-18 guard column (2 cm × 4 mm, 5 μm). The injection volume was 20 μl. Under these conditions the retention time of AA was about 4 min. AA concentration was determined from the corresponding calibration curve constructed with the pure chemical AA dissolved in the extraction solution.

2.4. Measurement of ^{14}C-AA cellular uptake

Uptake was stopped by washing the cells two times with ice-cold EB, containing an excess of unlabeled AA. Cells were then dissolved in 0.5 M NaOH and the incorporated radioactivity was measured by liquid scintillation spectrometry. The ^{14}C-AA non-specific binding to cells was assayed by performing the experiments at 0 °C. No differences between the controls (untreated samples) and 0 °C-^{14}C-AA-treated samples were detected.

2.5. Mitochondria isolation and subcellular fractionation

Mitochondria (M) were isolated either by differential centrifugation, as detailed in [27] or by sucrose density gradient centrifugation, as described by Koziel *et al.*, [28] with minor modifications. The latter method was also used to obtain the other sub-cellular fractions: nucleus (N), plasma membranes (PM), endoplasmic reticulum (ER) and cytosol (C). Briefly, cells were washed twice in PBS and resuspended in cold homogenization buffer (HB: 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 5 mM Tris–HCl, pH 7.4), in presence of protease inhibitor cocktail and digitonin (10 μg/ml) and homogenized using a glass potter in an ice-bath. The efficiency of homogenization was monitored under the microscope using trypan blue and stopped when the 90% of the total cells was disintegrated. The homogenate was centrifuged at 1000×*g* for 10 min at 4 °C and the supernatant (S1) was collected for the final centrifugation. The pellet was re-homogenized and the supernatant (S2) added to S1 and centrifuged at 20000×*g* for 20 min at 4 °C. The pellet obtained, containing nucleus fraction (*N*), was maintained at −20 °C till use. The supernatant was centrifuged at 100000×*g* for 60 min at 4 °C to obtain the endoplasmic reticulum fraction (*ER*, pellet) and the cytosol (*C*, supernatant). The pellet obtained from the centrifugation of S1 + S2 was resuspended in 5 mM MES, 0.2 mM EDTA pH 6.0 and separated by centrifugation at 20000×*g* for 2 h at 4 °C on a discontinuous 38%, 43% and 53% sucrose gradients. The middle density band corresponding to mitochondrial fraction (*M*) and the high density band corresponding to