J Bioenerg Biomembr (2006) 38:57–65
DOI 10.1007/s10863-006-9006-5

ORIGINAL PAPER

Identification and kinetic characterization of *Ht*DTC, the mitochondrial dicarboxylate–tricarboxylate carrier of Jerusalem artichoke tubers

Anna Spagnoletta · Aurelio De Santis · Elisabetta Tampieri · Elena Baraldi · Angela Bachi · Giuseppe Genchi

Received: 4 October 2005 / Accepted: 12 December 2006 / Published online: 20 June 2006

Abstract Jerusalem artichoke (*Helianthus tuberosus* L.) tubers were reported to be tolerant to cold and freezing. The aim of this study was to perform a kinetic characterization of the mitochondrial dicarboxylate–tricarboxylate carrier (*Ht*DTC) and to assess a possible involvement of this carrier in the cold tolerance of tubers. The *Ht*DTC was purified from isolated mitochondria by sequential chromatography on hydroxylapatite/celite and Matrex Gel Orange A. SDS gel electrophoresis of the purified fraction showed a single polypeptide band with an apparent molecular mass of 31.6 kDa. A polyclonal antibody raised against the tobacco DTC cross-reacted with the purified protein on Western blot analysis. In gel trypsin, digestion of the purified *Ht*DTC yielded peptides that exhibited strong amino acid sequence similarity to previously identified plant DTCs. Furthermore, using degenerate primers, a portion of the *Htdtc* cDNA was amplified and sequenced; this cDNA encoded for a protein with high sequence similarity to known plant homolog DTCs. When reconstituted in liposomes loaded with dicarboxylate (2-oxoglutarate, malate, malonate, succinate, and maleate) or tricarboxylate anions (citrate, *trans*-aconitate, and isocitrate), the purified *Ht*DTC transported all these anions in exchange with external [^{14}C]2-oxoglutarate. A kinetic characterization of *Ht*DTC was performed: (a) the half-saturation constant K_m and the V_{max} at 25°C of the 2-oxoglutarate/2-oxoglutarate exchange by reconstituted *Ht*DTC were found to be 360 μM and 10.9 μmol/(min mg protein), respectively; (b) the activation energy E_a of the succinate/2-oxoglutarate exchange by the reconstituted *Ht*DTC was found to be 50.7 kJ/mol constant between −5 and 35°C. Similarly, the activation energy E_a of succinate respiration of isolated Jerusalem artichoke mitochondria, measured between −2 and 35°C, was shown to be constant (65.3 kJ/mol). The physiological relevance of kinetic properties and temperature dependence of transport activities of *Ht*DTC is discussed with respect to the cold tolerance ability of Jerusalem artichoke tubers.

Keywords Mitochondria · Jerusalem artichoke · Dicarboxylate–tricarboxylate carrier · Cold tolerance

Abbreviations

ANT	adenine nucleotide translocator
DEPC	diethylpyrocarbonate
DTC	dicarboxylate–tricarboxylate carrier
JATM	Jerusalem artichoke tuber mitochondria
Pipes	1,4-piperazinediethanesulphonic acid
Taq	*Thermus aquaticus*
TBS	Tris buffer solution

A. Spagnoletta · G. Genchi (✉)
Dipartimento Farmaco–Biologico, Università della Calabria, Cosenza 87100, Italy
e-mail: genchi@unical.it

A. Spagnoletta · A. De Santis · E. Tampieri
Dipartimento di Scienze del Mare, Laboratorio di Fisiologia Vegetale, Università Politecnica delle Marche, Ancona, Italy

E. Tampieri · E. Baraldi
Dipartimento di Protezione e Valorizzazione Agro–Alimentare, Alma Mater Studiorum Università di Bologna, Bologna, Italy

A. Bachi
Mass Spectrometry Unit, Istituto Scientifico San Raffaele, Milano, Italy

Introduction

Jerusalem artichoke is a C3 crop with high yield capacity, showing high photosynthetic rates, capable of sustaining good growth rates in both normal and stressful environments (Schittenhelm, 1999). These features and the fact that Jerusalem artichokes tubers can be used as high fiber (i.e., inulin) food with no glucose and little fructose, has generated considerable interest in these plants as an alternative food source. Acclimated Jerusalem artichoke tubers are reported to be very useful materials for understanding the mechanism of cold and freezing tolerance. In particular, during the cold-acclimation process from September to January, the freezing tolerance of tubers (LT_{50}) increased from -2.8 to $-8.4^{\circ}C$ and the protoplasts isolated from acclimated tubers showed a consistent higher level of freezing tolerance (LT_{50} below $-25^{\circ}C$) (Murai and Yoshida, 1998).

Since the expression and activity of plant mitochondrial carriers are affected by abiotic stresses such as cold (De Santis et al., 1999; Maia et al., 1998) or ozone exposure (Kiiskinen et al., 1997) we decided to study the kinetic properties of mitochondrial metabolite carriers and the possible relationships of their activities with cold tolerance of Jerusalem artichoke tubers.

In fact, an efficient transport of a broad spectrum of dicarboxylates and tricarboxylates from cytosol into the mitochondrial matrix (and vice versa) is required for Krebs cycle activity and respiration, for the export (or import) of redox equivalents by metabolites shuttles, for fatty acids elongation and isoprenoid synthesis. It was demonstrated that in a wide variety of plant tissues these physiological functions were mainly operated by the expression and the activity of a plant specific carrier, the dicarboxylate–tricarboxylate carrier (DTC) (Picault et al., 2002). This protein is a member of the mitochondrial carrier family (MCF) (Picault et al., 2004).

Purification of the carrier from isolated mitochondria or from heterologous expression systems followed by reconstitution of the purified carrier in proteoliposomes has been commonly used for the functional characterization of transport proteins of this mitochondrial carrier family (Laloi, 1999).

In this paper, we have described the purification of dicarboxylate–tricarboxylate carrier (*Ht*DTC) from mitochondria isolated from Jerusalem artichoke tubers stored at 2°C, during their dormancy. A portion of the *Ht*dtc cDNA was amplified and sequenced and the kinetic properties of the purified DTC exchange reactions after incorporation into liposomes were investigated. To study the relationships between mitochondrial transport activity and cold tolerance of Jerusalem artichoke tubers, the temperature dependence of *Ht*DTC exchange activities and succinate respiration was also evaluated.

Materials and methods

Chemicals

[^{14}C]2-Oxoglutarate was purchased from Perkin-Elmer Life Sciences; hydroxyapatite (Bio-Gel HTP) from Bio-Rad; Triton X-100, *N*,*N*′-methylenebisacrylamide and acrylamide from Serva; egg-yolk phospholipids (phosphatidylcholine from fresh turkey egg-yolk) and Amberlite XAD-2 from Fluka; Matrex Gel Orange A from Amicon (Beverly, MA); cardiolipin, Pipes, SDS, and asolectin from Sigma; celite 535 from Roth and Sephadex G-75 from Amersham Biosciences. Taq polymerase from Fermentas (Burlington, Ont., Canada); Wizard SV Gel, PCR Clean Up system, plasmid pGEM-T Easy, and Im Prom II Reverse Transcription System from Promega (Madison, WI); T4 DNA ligase from NEB (Beverly, MA); Flexi Prep kit from Amersham (Little Chalfont, Buckinghamshire, UK); POROS R2 from Applied Biosystems (Foster City, CA); and CONCERT Plant Purification Reagent from Invitrogen (Carlsbad, CA). All other chemicals used were of analytical grade.

Isolation and purification of mitochondria

Mitochondria were isolated according to Spagnoletta et al. (2002) from tubers of a uniform population of Jerusalem artichoke (*Helianthus tuberosus* L.) harvested in late September and stored at 2°C in polyethylene bags. The purity of the mitochondrial preparations was checked by assaying marker enzymes for endoplasmic reticulum, peroxysomes, plasma membranes, and vacuoles, as described by Neuburger (1985). Finally, mitochondria were suspended at a concentration of 40–60 mg protein per mL of washing medium (pH 7.2), and used for respiration analysis or frozen in liquid nitrogen and stored at $-80^{\circ}C$, for subsequent *Ht*DTC purification.

Succinate oxidation of isolated mitochondria

Rate of oxygen consumption during succinate respiration by isolated JATM was measured at different selected temperatures with a Clark-type electrode (Dual Digital Model 20, Rank Brothers, Bottisham, Cambridge, England). The respiration medium contained 0.3 M sucrose, 5 mM Tris–Cl pH 7.2, 5 mM $MgCl_2$, 10 mM KCl, 2 mM KP_i, and 0.2 mM ATP. The medium pH was controlled at every temperature used. Maximum oxygen concentration values at every temperature were taken as reported in Clesceri et al. (1999).

Purification of the *Ht*DTC

The mitochondrial suspensions were centrifuged and pellets were solubilized in 3% Triton X-100 (w/v), 20 mM Na_2SO_4,

1 mM EDTA, and 10 mM Pipes, pH 7.0 (buffer A) at a final concentration of 15–18 mg protein per mL, according to Spagnoletta et al. (2002). After 15 min at 0°C the mixture was centrifuged. Then, 225 μL of this centrifuged supernatant, supplemented with 1.0 mg of cardiolipin in 25 μL of buffer A, were applied to cold hydroxyapatite/celite (1:2) columns and eluted with the same buffer. The first milliliter of the eluates from two hydroxyapatite/celite columns were pooled and applied onto a cold Matrex Gel Orange A column, pre-equilibrated with 4 mL of 0.1% Triton X-100, 10 mM Na_2SO_4, 1 mM EDTA, and 5 mM Pipes, pH 7.0 (buffer B). Elution was performed with 4 mL of buffer B, followed by 1.3 mL of buffer B in the presence of 2 mg/mL asolectin. Pure *Ht*DTC was collected in 800 μL, eluting with the second fraction of 800 μL of the buffer B in the presence of asolectin. The Matrex Gel Orange A had been previously activated washing sequentially with 4 mL of 8 M urea and distilled water until neutrality at room temperature, followed by 4 mL of buffer B at 4°C.

Purification steps of *Ht*DTC were followed by polyacrylamide slab gel electrophoresis of acetone-precipitated proteins in the presence of 0.1% SDS according to Laemmli (1970). A minigel system was used (8 cm × 10 cm × 1.5 mm). The stacking gel and the separation gel contained 5% and 17.5% acrylamide, respectively, with an acrylamide/bisacrylamide ratio of 150 to give a high resolution of polypeptides with molecular mass close to 30 kDa. Staining was performed by the silver nitrate method (Morrissey, 1981). Protein was determined by the Lowry method modified for the presence of Triton (Dulley and Grieve, 1975). All samples used for protein determination were subjected to acetone precipitation and dissolved in 1% SDS.

Immunoblotting of Purified *Ht*DTC

The proteins from the SDS polyacrylamide gel were electrotransferred to 0.45 μm supported nitrocellulose membrane (BioRad) for 1 h at 100 V and 4°C in a "Mini-Trans-Blot" electrophoretic transfer cell (BioRad). The membrane was incubated in blocking buffer containing 5% nonfat dry milk in TBS solution (25 mM Tris-HCl pH 7.5, 150 mM NaCl) for 1 h at room temperature, followed by incubation with rabbit polyclonal antibodies against *Nt*DTC (courtesy Dr. L. Palmieri) overnight at 4°C. Then, the membrane was washed four times in TBS containing 0.3% Tween 20, probed with the secondary antibody (horseradish-peroxidase-conjugated anti-rabbit Ig) in blocking buffer for 1 h at room temperature, and washed again in TBS containing 0.05% Tween 20. Detection of the signal immunocomplexes were performed by the peroxidase reaction (Galvez et al., 1996).

Partial cloning of *Ht*dtc cDNA

To clone the *Ht*dtc cDNA, total RNA was extracted from the parenchyma pith of Jerusalem artichoke tubers using the CONCERT Plant Purification Reagent. The dry RNA pellet was resuspended in DEPC water. To generate first strand cDNA 1 μg of total RNA was used in RT-PCR with the ImProm II Reverse Transcription System together with 0.5 μg oligo-d(T). First strand cDNA was used in PCR with degenerate primers annealing to conserved regions of known plant cDNA dtcs. Primer sequences were as follows: sense primer 5′-AAYGARGGNGTNGGNGCNTTYTAYAA-3′, antisense primer 5′-GTCATCATNACRTGNGGNGCDAT-3′. PCR reaction (50 μL) contained 0.1 μg of first strand cDNA, 0.4 μM of each primer, 200 μM dNTPs, 2 mM $MgCl_2$ and 1.5 U of Taq polymerase. PCR consisted of an initial denaturation step of 3 min at 95°C, followed by 35 cycles each of 1 min at 95°C, 45 s at 48°C, and 45 s at 72°C and a final extension step of 10 min at 72°C. PCR product was analyzed on agars gel. The band was excised, eluted, and purified using the Wizard SV Gel and PCR Clean Up System. PCR product was ligated into the plasmid pGEM-T Easy using T4 DNA ligase and used to transform electro competent DH5α *E. coli* cells. Plasmid DNA was isolated and purified from 3 mL liquid culture using the Flexi Prep kit. Two clones were sequenced by CRIBI (University of Padova, Italy) using universal primers (T7 and SP6). The obtained partial nucleotide sequence of putative *Ht*dtc cDNA was submitted to GenBank (accession number: AY700673).

Sequencing of peptides obtained by trypsin digestion of purified *Ht*DTC, in gel digestion and mass spectrometric analysis

Bands of interest containing purified *Ht*DTC were excised from the purification gel, subsequently reduced, alkylated, and digested overnight with bovine trypsin, as described in Shevchenko et al. (1996). Tryptic peptides were analyzed by Tandem MS experiments performed on a Q-Star pulsar (QqT of hybrid system from PE SCIEX Instrument, Toronto, Canada). Briefly, the protein digests were concentrated and desalted over a capillary column manually packed with 0.2 μL of POROS R2 material conditioned with 5% formic acid. The peptide mixture was eluted using 1 μL of 50% methanol/5% formic acid directly into the nanoelectrospray needle. Multiply charged peptides were fragmented in order to assign the amino acidic sequence.

For purified protein sequencing, the analysis by MALDI-TOF was followed by database search in MASCOT. The matched sequenced peptides were aligned to known DTC sequences using the software Clustal W (http://www.ebi.ac.uk/clustalw/).

Reconstitution of purified *Ht*DTC in proteoliposomes and transport measurements

Purified *Ht*DTC, eluted from Matrex Gel Orange A column, was reconstituted into liposomes as described in Spagnoletta et al. (2002). Transport was initiated by adding to proteoliposomes [^{14}C]2-oxoglutarate (0.1 mM final concentration). The exchange was stopped by adding 10 μL of 350 mM pyridoxal 5′-phosphate; in control samples, the inhibitor was added together with the labeled substrate at time zero. The assay temperature was 25°C. The external radioactivity was removed by passing the samples (150 μL) through a Sephadex G-75 column. The liposomes with internal radioactivity, eluted with 1.2 mL of 50 mM NaCl, were collected in 4 mL of scintillation cocktail, vortexed, and counted in a Tricarb 1600 TR liquid scintillation counter (Packard). The experimental values were corrected by subtracting the respective control. K_m and V_{max} values were determined by a computer-fitting program based on linear regression analysis.

Results

Purification of the DTC from isolated Jerusalem artichoke mitochondria

To purify the DTC, the mitochondrial membranes were first solubilized by treating JATM with Triton X-100 in the presence of salt and cardiolipin. Subsequently, the Triton extract was subjected to chromatography on hydroxyapatite/celite followed by a second chromatography on Matrex Gel Orange A (Table 1). The degree of purification of DTC was monitored at each chromatographic step both by SDS gel electrophoresis and by 2-oxoglutarate transport activity. The hydroxyapatite/celite chromatography led to a substantial purification of the DTC. About 95% of the solubilized mitochondrial proteins was bound to this resin, whereas about 76% of the total transport activity was recovered in the eluted fraction with a specific activity increase of 16-fold. The Matrex Gel Orange A eluted fraction, obtained by enriching the elution buffer with asolectin led to an increase in the specific activity of the reconstituted dicarboxylate–tricarboxylate carrier of 9.6-fold and 151-fold, with respect to that of the hydroxyapatite/celite eluate and of the mitochondrial extract, respectively. Approximately 26% of the total transport activity was recovered with a protein yield of 0.17%.

Table 1 Purification of the *Ht*DTC from Jerusalem artichoke mitochondria

Purification step	Protein (mg)	Specific activity	Total activity	Purification (fold)
Extract	6.50	63	409	1
Hydroxyapatite/celite	0.30	993	298	16
Matrex Gel Orange A[a]	0.011	9500	104	151

Note. The proteoliposomes were loaded with 20 mM 2-oxoglutarate and the exchange was started by the addition of 0.1 mM external [^{14}C]2-oxoglutarate. The activity of the reconstituted 2-oxoglutarate exchange is expressed in nmol/(10 min mg protein) (specific activity) and nmol/10 min (total activity).

[a] Eluate with buffer B plus asolectin (1.5 mL).

Figure 1 shows an SDS-polyacrylamide gel electrophoresis of mitochondrial extract (lane 1), hydroxyapatite/celite flow-through (lane 2), and Matrex Gel Orange A eluates (lanes 3, 4, and 6) obtained from JATM solubilized with Triton X-100. The fraction of lane 2 was substantially purified with respect to mitochondrial extract (lane 1), although it still contained various protein bands with apparent molecular masses from 20 and 40 kDa. The fraction of lane 6 shows that a single protein band with an apparent molecular mass of 31.6 kDa was eluted from Matrex Gel Orange A with buffer B and asolectin. The fractions of lanes 3 and 4 from Matrex Gel Orange A were not active in reconstituted 2-oxoglutarate exchange.

Identification of purified protein and cloning of *Ht*dtc cDNA

Immunoblot of the SDS purification gels using a *Nt*DTC polyclonal antibody showed cross-reactivity between the purified protein band and the *Nt*DTC polyclonal antibody (Fig. 2). A portion of the *Ht*dtc cDNA was cloned from Jerusalem artichoke tuber (GenBank, accession number: AY700673). This cDNA encoded a protein showing high sequence similarity to other known plant DTCs (Fig. 3). The obtained *Ht*DTC sequence was scored with peptides obtained with the NanoSpray ESI-MS/MS analysis and matches in three different regions were found (Fig. 3).

Activity of the purified *Ht*DTC reconstituted in proteoliposomes

Proteoliposomes were loaded with 20 mM cold 2-oxoglutarate and the time-course of the pyridoxal 5′-phosphate-sensitive [^{14}C]2-oxoglutarate uptake by proteoliposomes was measured (Fig. 4). The uptake of 0.1 mM [^{14}C]2-oxoglutarate was essentially linear at 25°C for about 5 min and then fell off and reached an equilibrium at a maximum value of about 22,000 nmol exchanged per mg protein after 90 min. There was no activity for liposomes incorporating the carrier protein denatured in boiling water for 2 min. Furthermore, no uptake of external [^{14}C]2-oxoglutarate was observed into proteoliposomes without internal 2-oxoglutarate, indicating that reconstituted *Ht*DTC do not catalyze unidirectional transport.

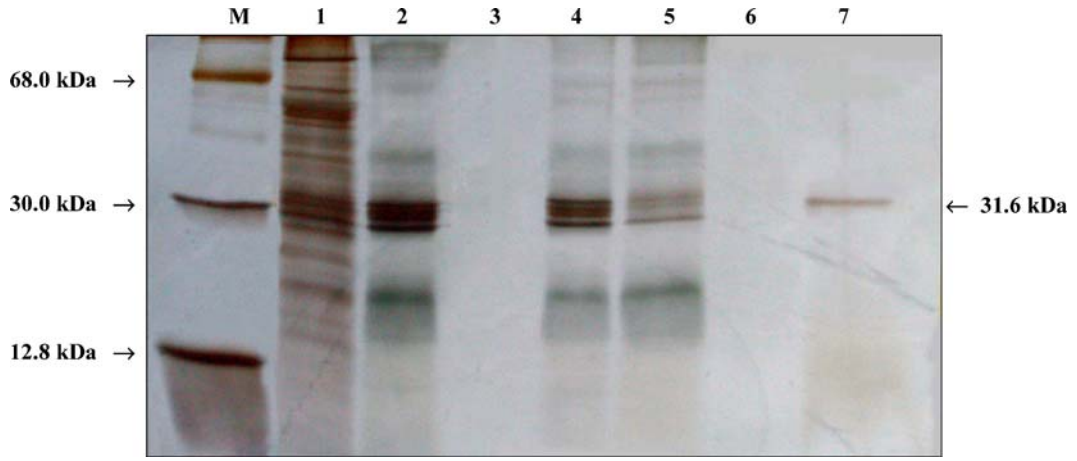

Fig. 1 Purification of dicarboxylate–tricarboxylate carrier (*Ht*DTC) from isolated Jerusalem artichoke mitochondria (JATM). SDS gel electrophoresis of fractions obtained by hydroxyapatite/celite and by Matrex Gel Orange A of JATM solubilized with Triton X-100. M, protein markers (from the top to the bottom: BSA, carbonic anhydrase and Cyt c); 1, Triton X-100 mitochondrial extract (150 μg in 20 μL); 2, hydroxyapatite/celite eluate (20 μg in 70 μL); 4, Matrex Gel Orange A pass-through (5.0 μg in 180 μL); 5, Matrex Gel Orange A eluate with buffer B (4.5 μg in 200 μL); 7, Matrex Gel Orange A eluate with buffer B plus asolectin (2.5 μg in 200 μL)

The time-course of [^{14}C]2-oxoglutarate/2-oxoglutarate exchange, as shown in the insert of Fig. 4, represents an exponential approach to isotopic equilibrium, which is demonstrated by the straight line obtained by plotting the natural logarithm of the equilibrium fraction (2-oxoglutarate$_{max}$/2-oxoglutarate$_{max}$-2-oxoglutarate) against time. This means that the influx of 2-oxoglutarate into proteoliposomes follows first-order kinetics. The first-order rate constant, k, extrapolated from the slope of the logarithmic plot was 0.08 min^{-1}.

The substrate specificity of the *Ht*DTC reconstituted protein was investigated by measuring the exchange of [^{14}C]2-oxoglutarate into liposomes loaded with a variety of substrates. In Table 2, it is shown that the highest activities were found with internal 2-oxoglutarate, malonate, malate, succinate, and maleate. Also citrate, isocitrate, and *trans*-aconitate were found to be exchanged for external radioactive 2-oxoglutarate, though to a slightly lower extent with respect to dicarboxylates. Labeled 2-oxoglutarate was not significantly exchanged against adipate, glutarate, pyruvate, glutamate, phosphate, and ATP.

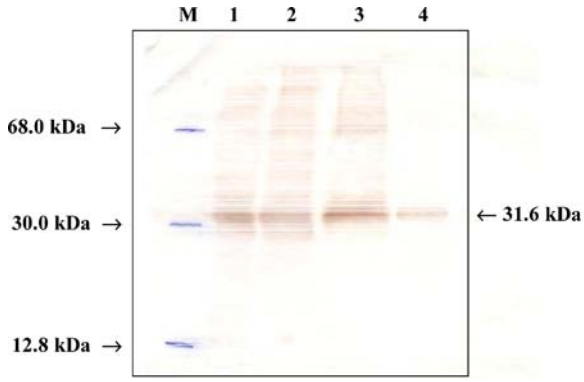

Fig. 2 Immunoblotting of purified *Ht*DTC. Binding of antibodies against *Nt*DTC to *Ht*DTC explored by Western blot analysis. Protein markers (lane M, from the top to the bottom: BSA, carbonic anhydrase and Cyt c), 400 μg of lysed mitochondria (lane 1), 225 μg of Triton X-100 mitochondrial extract (lane 2), 430 μg hydroxyapatite/celite eluate (lane 3), and 20 μg of Matrex Gel Orange A eluate with buffer B plus asolectin (lane 4) were electrophoresed on a 17.5% polyacrylamide gel. After being electroblotted, the nitrocellulose sheet was treated with the antiserum (1:1000 dilution). Immunodetection was performed as described under Materials and Methods section

The sensitivity of the reconstituted *Ht*DTC in proteoliposomes containing 20 mM 2-oxoglutarate to externally added substrates and inhibitors was also investigated (Tables 3 and 4). The [^{14}C]2-oxoglutarate/2-oxoglutarate exchange was strongly inhibited by 2-oxoglutarate, malate, malonate, succinate, maleate (about 90–95% inhibition), citrate, *trans*-aconitate, and isocitrate (about 70–80% inhibition). Phosphate, adipate, ATP, fumarate, glutamate, and pyruvate had a very low inhibitory effect (Table 3).

The *Ht*DTC activity was strongly inhibited by 10 mM pyridoxal 5′-phosphate and by 2 mM organic mercurials such as mersalyl, *p*-hydroxymercuribenzoate and *p*-chloromercuryphenylsulfonate (Table 4). Also 10 mM butylmalonate, benzylmalonate, and phtalate inhibited the reconstituted transport activity. Moreover, bathophenanthroline, 1,2,3-benzenetricarboxylate, and phenylisothiocyanate inhibited this carrier**,** though to a lower extent. In contrast, phenylglioxale, *N*-ethylmaleimide, and 2,3-pentanedione had no significant inhibitory effect.

Kinetic characteristics of reconstituted *Ht*DTC

The kinetic constants of reconstituted *Ht*DTC were determined by measuring the initial transport rate using various external [^{14}C]2-oxoglutarate concentrations and a constant internal concentration of 20 mM 2-oxoglutarate.

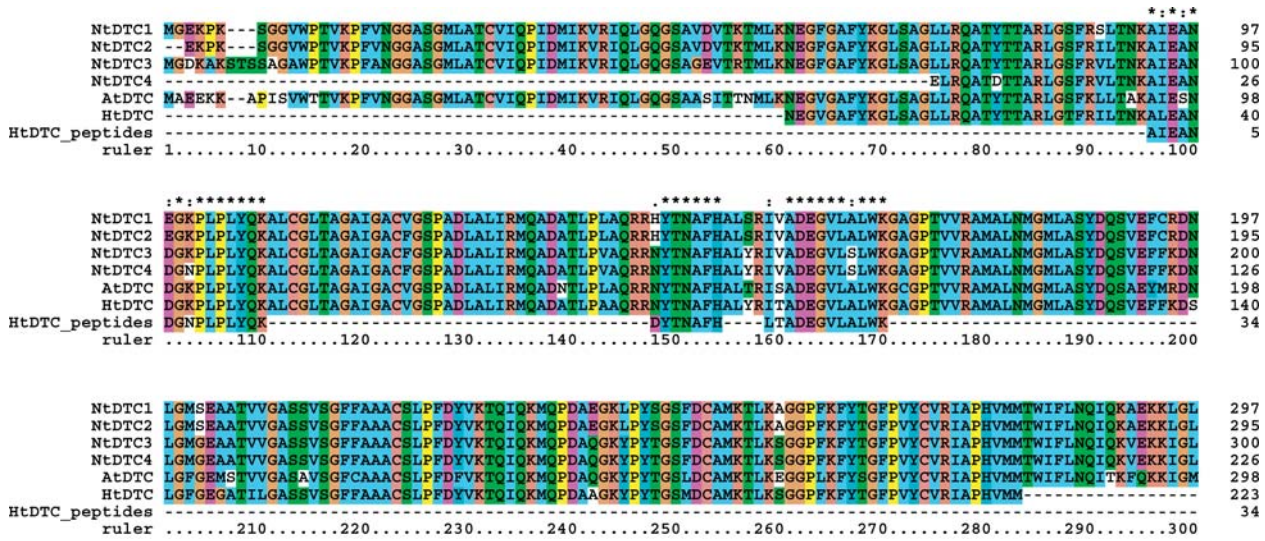

Fig. 3 Aligment of *Helianthus tuberosus* DTC sequence (*Ht*DTC, GenBank accession number: AY700673) with *Nicotiana tabacum* DTC sequences (*Nt*DTC1, *Nt*DTC2, *Nt*DTC3, and *Nt*DTC4), *Arabidopsis thaliana* DTC sequence (*At*DTC), and with peptides obtained from purified *Helianthus tuberosus* DTC. Conserved amino acids residues are represented with the same color. Identical residues in all sequences are indicated above sequences (*), whereas *dots* (., :) indicate the grade of similarity. *Dashes* represent gaps in the sequence

Five different exchange measurements were performed at 25°C and an average K_m value of $358 \pm 45\,\mu$M and a V_{max} of $10.9 \pm 1.60\,\mu$mol/(min mg protein) were calculated. Citrate, succinate, and malate were found to be competitive inhibitors of 2-oxoglutarate transport mediated by *Ht*DTC, since they increased the apparent K_m without changing the V_{max} of the [^{14}C]2-oxoglutarate/2-oxoglutarate exchange. The inhibition constants (K_i) were $0.10 \pm 0.030\,\mu$M for citrate, $0.13 \pm 0.033\,\mu$M for succinate, and $0.20 \pm 0.060\,\mu$M for malate (mean of three experiments).

Rates of transport exchanges by reconstituted *Ht*DTC were temperature-dependent. In an Arrhenius plot, continuous straight lines were obtained in the range from −5 to 35°C for [^{14}C]2-oxoglutarate/succinate exchange (Fig. 5) and for [^{14}C]2-oxoglutarate/2-oxoglutarate exchange (not shown). The activation energy (E_a) for [^{14}C]2-oxoglutarate/succinate exchange, as derived from the slope is 50.7 kJ/mol, in the

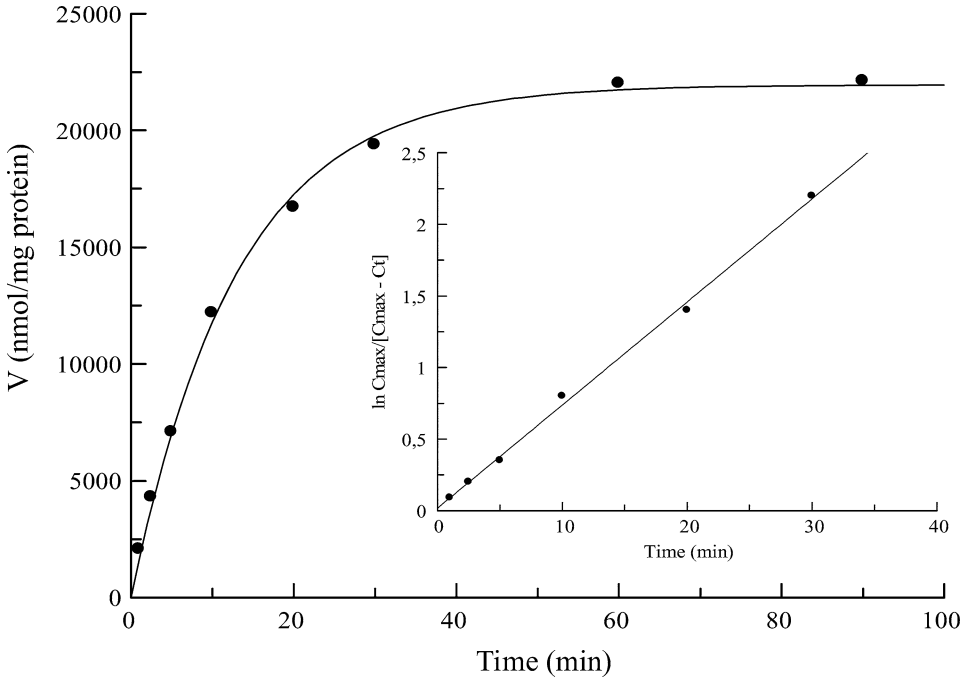

Fig. 4 Time-course of 2-oxoglutarate uptake in proteoliposomes reconstituted with purified *Ht*DTC. [^{14}C]2-oxoglutarate (0.1 mM) was added at zero time to proteoliposomes containing 20 mM 2-oxoglutarate. The insert represents the logarithmic plot of ln [2-oxoglutarate$_{max}$/(2-oxoglutarate$_{max}$ − 2-oxoglutarate$_t$)], where 2-oxoglutarate$_{max}$ is the maximum 2-oxoglutarate exchange per mg protein and 2-oxoglutarate$_t$ is the 2-oxoglutarate exchange per mg protein at time t, according to the relation ln 2-oxoglutarate$_{max}$/(2-oxoglutarate$_{max}$ − 2-oxoglutarate$_t$) = kt

Table 2 Rates of influx of external 2-oxoglutarate in exchange with different internal substrates, preloaded in proteoliposomes

Internal substrate (20 mM)	Rate of 2-oxoglutarate influx (nmol/(10 min mg protein)
None (Cl^- present)	470
2-Oxoglutarate	9810
Malonate	8830
Malate	8563
Succinate	8080
Maleate	6833
Citrate	6250
Isocitrate	5840
trans-Aconitate	5580
Oxoadipate	2354
Glutarate	1255
Pyruvate	1150
Adipate	961
Glutamate	780
Phosphate	600
ATP	590

Note. The proteoliposomes were loaded with the indicated substrates. Transport was initiated by adding external 0.1 mM [^{14}C]2-oxoglutarate. The results are the mean of three experiments.

indicated temperature range. A linear temperature dependence was also obtained by plotting the rate of succinate respiration of whole isolated JATM (Fig. 5) in an Arrhenius plot (from −2 to 35°C). The activation energy (E_a) of the respiratory activity of this substrate was found to be 65.3 kJ/mol.

Table 3 Sensitivity of 2-oxoglutarate/2-oxoglutarate exchange in proteoliposomes to externally added substrates

Addition	Inhibition (%)
2-Oxoglutarate	96
Malate	95
Malonate	94
Succinate	94
Maleate	91
Citrate	80
trans-Aconitate	73
Isocitrate	70
Oxoadipate	63
Phosphate	15
Adipate	11
ATP	10
Fumarate	10
Glutamate	10
Pyruvate	8

Note. The proteoliposomes were loaded with 20 mM 2-oxoglutarate and the exchange was started by adding 0.1 mM [^{14}C]2-oxoglutarate. The external substrates were added together with [^{14}C]2-oxoglutarate at a final concentration of 2.0 mM. The control values of uninhibited 2-oxoglutarate exchange was 10,130 nmol/(10 min mg protein). The data are the mean of three experiments.

Table 4 Sensitivity to inhibitors of 2-oxoglutarate/2-oxoglutarate exchange in proteoliposomes

Addition	Concentration (mM)	Inhibition (%)
Pyridoxal 5′-phosphate	10.0	96
Butylmalonate	10.0	91
p-I-Benzylmalonate	10.0	76
Phtalate	10.0	81
Mersalyl	2.0	95
p-Hydroxymercuribenzoate	2.0	90
p-Chloromercuryphenylsulfonate	2.0	87
Bathophenanthroline	10.0	65
1,2,3-Benzenetricarboxylate	10.0	60
Phenylisothiocyanate	10.0	46
Phenylglioxale	10.0	16
N-Ethylmaleimide	2.0	13
2,3-Pentanedione	10.0	10

Note. The proteoliposomes were loaded with 20 mM 2-oxoglutarate and the exchange was started by adding 0.1 mM [^{14}C]2-oxoglutarate. The inhibitors were added together with [^{14}C]2-oxoglutarate except that the −SH reagents were added 2 min before the labeled substrate. The control values of uninhibited 2-oxoglutarate exchange was 9,780 nmol/(10 min mg protein). The data are the mean of three experiments.

Discussion

We have purified a 31.6 kDa protein from JATM which, when reconstituted in liposomes, was able to activate the transport of dicarboxylates and tricarboxylates. The conclusion that the polypeptide of 31.6 kDa, which we have purified from JATM, is in fact the *Ht*DTC is supported by the following evidence. The polyclonal antibody against *Nt*DTC showed a cross-reaction with the purified 31.6 kDa protein. Sequences of peptides obtained from the purified 31.6 kDa protein were either identical or highly similar to the amino acid sequences of *Nt*DTC, *At*DTC (Picault et al., 2002), and to the deduced amino acid sequences of *Ht*DTC (present work). Upon reconstitution into liposomes, the purified carrier catalyzed a very active [^{14}C]2-oxoglutarate/2-oxoglutarate exchange. The purified transporter exhibited a substrate specificity and an inhibitor sensitivity similar to those observed for the DTCs in mitochondria from other plant sources (Picault et al., 2002).

The *Ht*DTC protein may play an important role during the Jerusalem artichoke tuber growth and dormancy. The citrate exported from the mitochondria to the cytosol in exchange for oxaloacetate can be cleaved by citrate lyase (Kaethner and Ap Rees, 1985) to acetyl-CoA and oxaloacetate and used for fatty acid elongation (Ohlrogge and Brause, 1995) and isoprenoid synthesis (Mc-Garvey and Croteau, 1995). The efflux of citrate, isocitrate, or 2-oxoglutarate in exchange for malate or oxaloacetate may also be involved in other metabolic processes, such as nitrate assimilation (Hanning and Heldt, 1993) and amino acid biosynthesis, which require

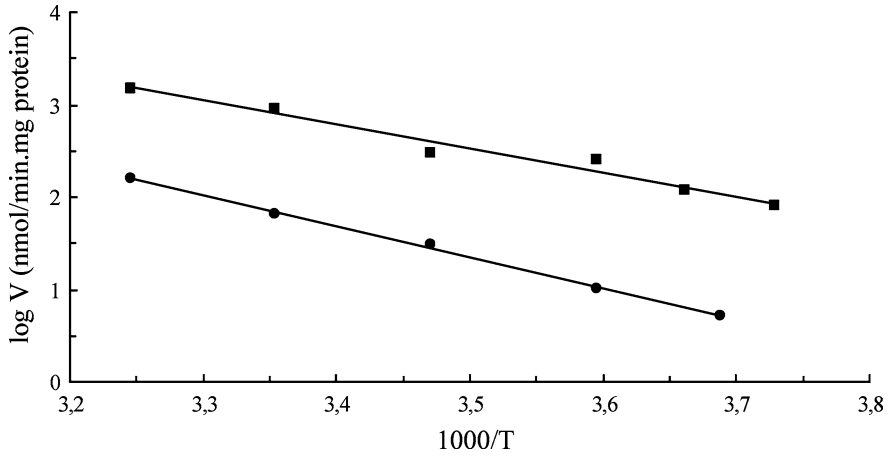

Fig. 5 Arrhenius plots of temperature dependence of 2-oxoglutarate/succinate exchange activity by purified *Ht*DTC reconstituted in proteoliposomes and of succinate respiration by whole mitochondria isolated from Jerusalem artichoke tubers. [^{14}C]2-oxoglutarate (0.1 mM) was added to proteoliposomes containing 20 mM succinate, which were incubated for 1 min at the indicated temperatures and rates of exchange were measured (■). Oxygen consumption rates were measured in a temperature range from −2 to 35°C (●). Mitochondria (0.8 mg protein) were suspended in 1 mL of respiration medium (see Materials and Methods section) with the addition of 5 mM Tris succinate and 1 mM ADP. The means ± SD of three experiments are reported. Maximal standard deviations were 0.01 nmol/(min mg)

production of 2-oxoglutarate in the cytosol. Thus, under these conditions, citrate and isocitrate exported from the mitochondria by the *Ht*DTC can be converted to 2-oxoglutarate by the cytosolic enzymes aconitase (Wendel et al., 1988) and isocitrate dehydrogenase, and then to glutamate (Chen and Gadal, 1990) by glutamine synthetase and glutamate synthase system (Sasakibara et al., 1998; Sukanja et al., 1994). A further physiological significance for the *Ht*DTC may be the transfer of reducing equivalents from the mitochondrial matrix to the cytosol by catalyzing a malate/oxaloacetate exchange, as previously proposed in mammalian and plant mitochondria (Gimpel et al., 1973; Krömer and Heldt, 1991; Passarella et al., 1977).

The kinetic characterization of the reconstitutively active *Ht*DTC is an important step toward investigations of the involvement of the activity of this carrier in the cold tolerance mechanisms in Jerusalem artichoke tubers. It was previously demonstrated that the activity of the maize ADP/ATP carrier protein (ANT) was sensitive to chilling stress and the level of this sensitivity was dependent on the different chilling tolerance shown by selected populations of maize (De Santis et al., 1999). Moreover, the activation energy of the ATP/ATP exchange in liposomes reconstituted with ANT purified from maize mitochondria was found temperature dependent, showing a biphasic behavior (22 kJ/mol between 35 and 15°C and 48 kJ/mol between 15 and 0°C) (Genchi et al., 1996). The break point at 15°C is near to the limit growth temperature of maize (about 12°C).

On the other hand, we have demonstrated that both succinate and 2-oxoglutarate (not shown) exchange catalyzed by purified *Ht*DTC in reconstituted liposomes, as well as succinate respiration by isolated JATM**,** showed a linear temperature dependence in Arrhenius plots, until −5 and −2°C, respectively. Consequently, on the basis of these findings we suggest that metabolite carriers activities contribute to cold tolerance for those plant tissues or organs that exhibit this behavior. Work in this direction is now in progress in our laboratories.

Acknowledgments This research was supported by grants from Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2003, MIUR, Italy), from Università della Calabria (Cosenza, Italy) and from Università Politecnica delle Marche (Ancona, Italy).

Bibliography

Chen RD, Gadal P (1990) Plant Physiol Biochem 28:141–145
Clesceri LS, Greenberg AE, Eaton AD (1999) Standard Methods for the Examination of Water and Wastewater (20th edition) American Public Health Association, American Water Works Association and Water Environment Federation Publishers, Washington, DC, p. 4–132
De Santis A, Landi P, Genchi G (1999) Plant Physiol 119:743–754
Dulley IR, Grieve PA (1975) Anal Biochem 64:136–141
Galvez S, Hodges M, Decottignies P, et al (1996) Plant Mol Biol 30:307–320
Genchi G, Ponzone C, Bisaccia F, et al (1996) Plant Physiol 112:845–851
Gimpel JA, De Haan EJ, Tager JM (1973) Biochim Biophys Acta 292:582–591
Hanning I, Heldt HW (1993) Plant Physiol 103:1147–1154
Kaethner TM, ap Rees T (1985) Planta 163: 290–294
Kiiskinen M, Korhonen M, Kangasjarvi J (1997) Plant Mol Biol 35:271–279
Krömer S, Heldt HW (1991) Biochim Biophys Acta 1057:42–50
Laemmli K (1970) Nature 227:680–685

Laloi M (1999) Cell Mol Life Sci 56:918–944
Maia IG, Benedetti CE, Leite A, et al (1998) FEBS Lett 429: 408–406
McGarvey DJ, Croteau R (1995) Plant Cell 7:1015–1026
Morrissey JH (1981) Anal Biochem 117:307–310
Murai M, Yoshida S (1998) Plant Cell Physiol 39:97–105
Neuburger M (1985) In Encyclopedia of Plant Physiology, Vol 18 (New Series) Preparation of Plant Mitochondria (Douce R, and Day DA, eds) Springer Verlag New York pp 1–24
Ohlrogge J, Brause J (1995) Plant Cell 7:957–970
Passarella S, Palmieri F, Quagliariello E (1977) Arch Biochem Biophys 180:160–168
Picault N, Hodges M, Palmieri L, Palmieri F (2004) Trends Plant Sci 9:138–146
Picault N, Palmieri L, Pisano I, et al (2002) J Biol Chem 277:24204–24211
Sasakibara H, Kawabata S, Takahashi H, et al (1998) Plant Cell Physiol 33:49–58
Schittenhelm S (1999) Crop Sci 39:1815–1823
Shevchenko A, Wilm M, Vorm O, Mann M (1996) Anal Chem 68:850–858
Spagnoletta A, De Santis A, Palmieri F, et al (2002) J Bioenerg Biomembr 34:465–472
Sukanja R, Li MG, Snustad DP (1994) Plant Mol Biol 26:1935–1946
Wendel JF, Goodman MM, Stuber CW, et al (1988) Biochem Genet 26:421–445