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Purification and Characterization of the Reconstitutively Active Adenine Nucleotide Carrier From Mitochondria of Jerusalem Artichoke (*Helianthus Tuberosus* L.) Tubers

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The adenine nucleotide carrier from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers mitochondria was solubilized with Triton X-100 and purified by sequential chromatography on hydroxapatite and Matrex Gel Blue B in the presence of cardiolipin and asolectin. SDS gel electrophoresis of the purified fraction showed a single polypeptide band with an apparent molecular mass of 33 kDa. When reconstituted in liposomes, the adenine nucleotide carrier catalyzed a pyridoxal 5′-phosphate-sensitive ATP/ATP exchange. It was purified 75-fold with a recovery of 15% and a protein yield of 0.18% with respect to the mitochondrial extract. Among the various substrates and inhibitors tested, the reconstituted protein transported only ATP, ADP, and GTP and was inhibited by bongkrekate, phenylisothiocyanate, pyridoxal 5′-phosphate, mersalyl and *p*-hydroxymercuribenzoate (but not *N*-ethylmaleimide). Atractyloside and carboxyatractyloside (at concentrations normally inhibitory in animal and plant mitochondria) were without effect in Jerusalem artichoke tubers mitochondria. V_{max} of the reconstituted ATP/ATP exchange was determined to be 0.53 μmol/min per mg protein at 25°C. The half-saturation constant K_m and the corresponding inhibition constant K_i were 20.4 μM for ATP and 45 μM for ADP. The activation energy of the ATP/ATP exchange was 28 KJ/mol between 5 and 30°C. The N-terminal amino acid partial sequence of the purified protein showed a partial homology with the ANT protein purified from mitochondria of maize shoots.

KEY WORDS: Adenine nucleotide; Mitochondria; Jerusalem artichoke; ATP; ADP; GTP.

INTRODUCTION

The inner membrane of plant mitochondria contains at least 10 specific carrier systems for the transport of metabolites (Day and Wiskich, 1984; Douce and Neuburger, 1989; Earnshaw, 1977; Hanson, 1985; Heldt and Flügge, 1987; Oliver 1987; Pozueta-Romero *et al.*, 1991). The main properties of these carriers have all been studied in intact mitochondria. However, essential for the identification of a transport protein and for its detailed functional and structural characterization is the purification and reconstitution of the purified protein in artificial membranes. So far, six of the plant mitochondrial metabolite carriers have been partially purified and reconstituted into liposomes, namely the dicarboxylate (Vivekananda *et al.*, 1988), glutamate/aspartate (Vivekananda and Oliver, 1989), monocarboxylate (Vivekananda and Oliver, 1990), tricarboxylate (McIntosh and Oliver, 1992) and phosphate (McIntosh and Oliver, 1994) carriers from pea seedlings, and α-ketoglutarate (Genchi *et al.*, 1991) from maize shoots. The ADP/ATP carrier (Genchi *et al.*, 1996) and the citrate carrier (Genchi *et al.*, 1999) from maize shoot mitochondria and the phosphate carrier from potato mitochondria

Key to abbreviations: ANT, adenine nucleotide transporter; JATM, Jerusalem artichoke tuber mitochondria; MTP, mitochondrial transition pore; Pipes, 1,4-piperazinediethanesulphonic acid; PVDF, polyvinylidene difluoride; SDS, sodium dodecylsulfate.

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(Silva *et al.*, 1999) have been purified to homogeneity and kinetically characterized. Moreover, the malate translocator (Taniguchi and Sugiyama, 1996, 1997), the phosphate carrier (Takabatake *et al.*, 1999), the uncoupling protein (PUMP) (Boreky *et al.*, 2001), and the plant mitochondrial dicarboxylate–tricarboxylate carrier (DTC) (Picault *et al.*, 2002) have been identified from their transport properties upon overexpression in *E. coli* or *S. cerevisiae* and reconstitution into liposomes.

The ADP/ATP carrier (adenine nucleotide transporter, ANT) is an intrinsic protein of the inner mitochondrial membrane which exchanges cytoplasmic ADP for ATP synthesized inside the mitochondrion. This reaction is essential for the transfer of energy from oxidative phosphorylation to extramitochondrial processes (Klingenberg, 1985; Vignais *et al.*, 1985). The ANT protein from bovine heart was reported to be reversibly converted into a large channel by Ca^{2+} ions, showing properties similar to the mitochondrial transition pore (MTP) (Brustovetsky and Klingenberg, 1996). It was demonstrated that MTP plays an important role in cell physiology and pathophysiology and most strikingly was involved in programmed cell death (Bernardi *et al.*, 2001).

The ANT1 protein purified from maize shoots can transport ATP, ADP, and also GTP, GDP, and deoxy-ATP (Genchi *et al.*, 1996). Thus, its active center is similar to, but not identical with the ANT protein of rat heart mitochondria.

Recently, it was reported that the identification and functional reconstitution of ANT1p, a peroxisomal transporter in the yeast *Saccharomyces cerevisiae*, which has the characteristic sequence features of the mitochondrial carrier family, showed different substrate and different inhibitor specificities from those of the heart mitochondrial ANT (Palmieri, 2001), since it can transport deoxynucleotides.

It was previously suggested that ANT activity is differently affected by chilling temperatures in maize plants obtained from populations selected for high and low germination levels at 9.5°C (De Santis *et al.*, 1999). Jerusalem artichoke tuber tissues were reported to be not only chilling-resistant but also freezing-resistant (Murai and Yoshida, 1998a,b). ATP/ADP exchange was reported to be atractyloside and carboxyatractyloside-insensitive in whole Jerusalem artichoke tuber mitochondria (JATM) (Passam and Coleman, 1975).

To continue the study of these properties of different ANT proteins in animal, plant, and yeast cells, we describe here the purification of the ADP/ATP carrier from JATM, using functional reconstitution as a monitor of the carrier activity during isolation. Upon SDS gel electrophoresis the purified JATM ANT protein appears to be a single polypeptide with an apparent molecular mass of 33 kDa. We have also determined the partial N-terminal sequence of the first 10 amino acids of the purified carrier. The functional properties of the JATM ANT protein incorporated into liposomes are also described here.

MATERIALS AND METHODS

Chemicals

Hydroxyapatite (Bio-gel HTP) was purchased from Bio-Rad; egg-yolk phospholipids (phosphatidylcholine from fresh turkey egg yolk), Dowex AG1-X8 (100–200 mesh), and Amberlite XAD-2 from Fluka; Triton X-100, acrylamide, and N,N′-methylenebisacrylamide from Serva; Matrex Gel Blue B from Amicon (Beverly, MA); [^{3}H]ATP from Amersham; cardiolipin from Avanti-Polar Lipids (Alabaster, AL); and Sephadex G-75 from Pharmacia. All other reagents were analytical grade.

Isolation and Purification of Mitochondria

Mitochondria were isolated from the tubers of Jerusalem artichoke (*Heliantus tuberosus* L.) according to the rapid method of Palmer (1967) with some modifications. With a plastic vegetable grater, 500 g of peeled Jerusalem artichoke tubers were grated into 1 L of ice-cold medium containing 0.5 M sucrose, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% (w/v) defatted BSA, 0.05% (w/v) cysteine. Grated tissue was further disrupted with a Braun mixer in the same medium for approximately 90 s with some intervals. The homogenate was filtered through a layer of nylon sheet (Saracilene T200, Gaudenzi Tecnica Industriale, Padova, Italy) and centrifuged for 10 min at 10,000*g*. The pellet was resuspended in 50 mL of washing medium containing 0.4 M sucrose, 5 mM Tris-HCI, pH 7.2, and centrifuged for 5 min at 1,000*g*. For the isolation of the mitochondria, the resultant supernatant was centrifuged at 8,000*g* for 10 min. The mitochondria were then resuspended at a cencentration of 15–18 mg protein per mL of washing medium (pH 7.2), frozen in liquid nitrogen and stored at −80°C. The purity of the mitochondrial preparation was checked by assaying marker enzymes for endoplasmic reticulum, peroxysomes, plasma membranes, and vacuoles, as described by Neuburger (1985).

Purification of the ADP/ATP Carrier

Jerusalem artichoke tuber mitochondria 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. After 15 min at 0°C the mixture was centrifuged at 15,000*g* for 20 min; then 225 μL of ultracentrifuged supernatant (Triton extract), supplemented with cardiolipin (1 mg in 25 μL of buffer A), were applied to cold hydroxyapatite columns (Pasteur pipettes containing 600 mg of dry material) and eluted with the extraction buffer. The first milliliters of the eluates from two hydroxyapatite columns were pooled and applied on cold Matrex Gel Blue B columns (Pasteur pipettes containing 1 mL of resin), preequilibrated with 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 3 mL of buffer B, followed by 1.3 mL (divided into two fractions: a first fraction of 500 μL and a second fraction of 800 μL) of buffer B supplemented with 6 mg/mL asolectin. Pure ADP/ATP carrier 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 Blue B had been previously washed sequentially with 4 mL of 8 M urea/0.5 M NaOH, 4 mL of distilled water, and 4 mL of buffer B. The urea/base wash and the water wash were carried out at room temperature, whereas the buffer B wash was performed in a cold room. All the other operations were performed at 4°C.

Reconstitution of the ADP/ATP Carrier in Liposomes

Protein eluates were reconstituted by removing the detergent with a hydrophobic column (Palmieri *et al.*, 1995). In this procedure, the mixed micelles containing detergent, protein, and phospholipids were repeatedly passed through the same Amberlite XAD-2 column. The composition of the initial mixture used for reconstitution was (a) 200 μL of the eluates of the different columns or 20 μL of the Triton extract plus 180 μL of buffer A; (b) 100 μL of 10% Triton X-114; (c) 100 μL of 10% egg-yolk phospholipids in the form of sonicated liposomes (Bisaccia *et al.*, 1985); (d) 20 mM ATP or other substrates as indicated in the legends to figures and tables; and (e) 10 mM Pipes, pH 7.0, in a final volume of 700 μL. After vortexing, this mixture was passed 15 times through the same Amberlite column (0.5 × 3.6 cm) preequilibrated with a buffer containing 10 mM Pipes, pH 7.0, and 20 mM of the substrates present in the starting mixture. All the operations were performed at 4°C, except the passage through the Amberlite column, which was carried out at room temperature.

Transport Measurements

To remove the external substrate, 650 μL of the proteoliposomal suspension were passed through a Sephadex G-75 column (0.7 × 15 cm) preequilibrated with 50 mM NaCl and 10 mM Pipes, pH 7.0. The eluted proteoliposomes (600 μL), distributed in reaction vessels (150 μL), were used for transport measurements by the inhibitor stop method (Palmieri and Klingenberg, 1979). Transport was started by adding 10 μL of [^{3}H]ATP at the indicated concentrations. The exchange was stopped, after the desired time interval, by adding 10 μL of 350 mM pyridoxal 5′-phosphate. In control samples the inhibitor was added together with the labeled substrate. The assay temperature was 25°C. The external radioactivity was removed by passing the samples (160 μL) through an anion-exchange column (Dowex AG1-X8, acetate form, 0.5 × 5 cm). The liposomes eluted with 1.0 mL of 50 mM sodium acetate were collected in 4 mL of scintillation mixture, vortexed and counted. The experimental values were corrected by substracting the respective control. The pyridoxal 5′-phosphate-insensitive radioactivity in the control samples was always less than 5–6% of the pyridoxal 5′-phosphate-sensitive radioactivity taken up during the transport assay. K_m and V_{max} values were determined by a computer-fitting program based on linear regression analysis.

Other Methods

Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli (1970). A minigel system was used; gel sizes were 8 cm × 10 cm × 1.5 mm (thickness). The stacking gel contained 5% acrylamide and the separation gel contained 17.5% acrylamide with a ratio acrylamide/bisacrylamide of 30:0.2 to give a high resolution of polypeptides with a molecular mass close to 30 kDa. Staining was performed by the silver nitrate method (Morrissey, 1981). For protein sequencing the pure protein separated with the same electrophoretic system was transferred to a PVDF membrane, detected by staining with Coomassie Brilliant Blue, excised and subjected to Edman degradation in an Applied Biosystems 477A pulse liquid protein sequencer equipped with an on-line phenylthiohydantoin-amino acid analyzer. 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 redissolved in 1% SDS.

RESULTS

Purification of the Adenine Nucleotide Carrier

JATM were solubilized in Triton X-100 in the presence of cardiolipin and subjected to chromatography on

Table I. Purification of the Adenine Nucleotide Carrier From *Helianthus Tuberosus* Mitochondria

Purification step	Protein (mg)	Specific activity	Total activity	Purification (fold)
Extract	6.80	45	306	1
Hydroxyapatite	0.30	730	219	16
Matrex Gel Blue B[a]	0.012	3400	41	76

Note. The proteoliposomes were loaded with 20 mM ATP and the exchange was started by the addition of 0.1 mM external [^{3}H]ATP. The activity of the reconstituted ATP exchange is expressed in nmol/10 min per mg protein (specific activity) and nmol/10 min (total activity).
[a] Eluate with buffer B plus asolectin (1.5 mL).

hydroxyapatite followed by a second chromatography on Matrex Gel Blue B (Table I). The passage of the mitocondrial extract through hydroxyapatite led to a substantial purification of the adenine nucleotide carrier. About 95% of the proteins present in the extract were bound to this resin. In the hydroxyapatite eluate 72% of the total activity was recovered and the specific activity was increased 16-fold. For further purification, the hydroxyapatite pass-through was subjected to chromatography on Matrex Gel Blue B (see Methods). By this purification step, eluting the column with buffer B in the presence of asolectin, the specific activity of the reconstituted ATP transport was increased 4.7-fold and 76-fold with respect to that of the hydroxyapatite eluate and of the mitochondrial extract respectively. Approximately 13% of the total transport activity was recovered with a protein yield of 0.18%.

Figure 1 shows an SDS-polyacrylamide gel electrophoresis of hydroxyapatite pass-through (Lane 2) and Matrex Gel Blue B eluate (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 28 to 36 kDa. Figure 1 (Lane 6) shows that a single protein band with an apparent molecular mass of 33 kDa was eluted from Matrex Gel Blue B in the presence of asolectin. The fractions of Lanes 3 and 4 of Matrex Gel Blue B were not active in reconstituted ATP transport.

Properties of the Reconstituted Adenine Nucleotide Carrier

In all the experiments shown below, the reconstituted system consisted of purified protein eluted in the presence of asolectin from Matrex Gel Blue B (Lane 6) and incorporated into liposomes. Figure 2 illustrates the time-course of the pyridoxal 5′-phosphate-sensitive [^{3}H]ATP uptake by proteoliposomes which were loaded with unlabelled ATP (20 mM). The uptake of ATP increased linearly with time for about 5 min at a rate of 1400 nmol/min per mg protein

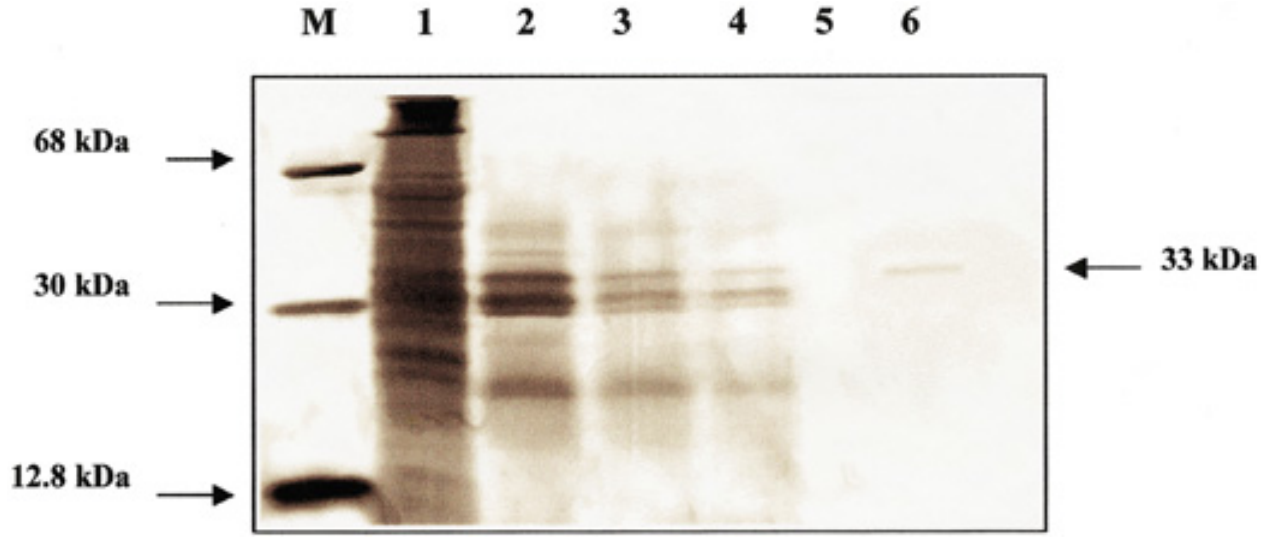

Fig. 1. Purification of ADP/ATP carrier from JATM. SDS gel electrophoresis of fractions obtained by hydroxyapatite and by Matrex Gel Blue B of JATM solubilized with Triton X-100. M, protein markers (from the top to the bottom: bovine serum albumin, carbonic anhydrase, and cytochrome *c*); 1, Triton X-100 mitochondrial extract (180 μg in 15 μL); 2, hydroxyapatite eluate (26 μg in 80 μL); 3, Matrex Gel Blue B pass-through (5.1 μg in 200 μL); 4, Matrex Gel Blue B eluate with buffer B (4.5 μg in 200 μL); 6, Matrex Gel Blue B eluate with buffer B plus asolectin (2 μg in 200 μL).

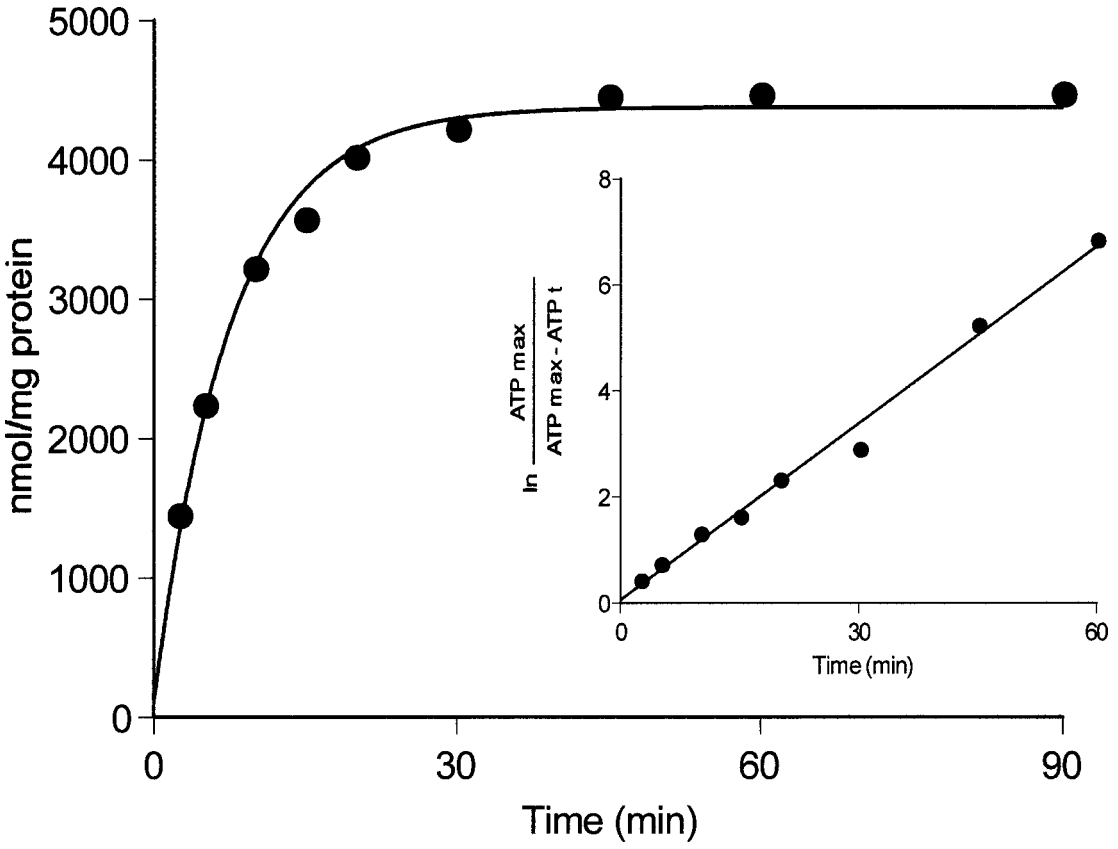

Fig. 2. Time-course of ATP uptake in reconstituted liposomes. A total of 0.1 mM [^{3}H] ATP was added at zero time to reconstituted liposomes containing 20 mM ATP. The insert represents the logarithmic plot of In $ATP_{max}/(ATP_{max} - ATP_t)$, where ATP_{max} is the maximum ATP exchange per mg protein and ATP_t the ATP exchange per mg protein at time t, according to the relation In $ATP_{max}/(ATP_{max} - ATP_t) = k_t$.

at 25°C (at 0.1 mM [^{3}H]ATP). In the absence of internal substrate, ATP uptake was totally absent. Likewise, there was no activity without incorporation of the carrier protein or with incorporation of heat-denatured carrier protein (2 min at 100°C) into the liposomes.

The time-course of [^{3}H]ATP/ATP exchange, as shown in the insert of Figure 2, represents an exponential approach to isotopic equilibrium, which is demonstrated by the straight line obtained by plotting the natural logarithm of the fraction of equilibrium $ATP_{max}/(ATP_{max} - ATP_t)$ against time. This means that the exchange of ATP in proteoliposomes follows first-order kinetics. The first-order rate constant, k, extrapolated from the slope of the logarithmic plot was 0.11 min^{-1}.

The rate of ATP/ATP exchange is temperature dependent. In an Arrhenius plot, a straight line was obtained in the range from 5 to 30°C (results not shown). Above this temperature the ADP/ATP carrier is completely denaturated. The activation energy as derived from the slope was 28 kJ/mol.

The substrate specificity of [^{3}H]ATP with respect to intraliposomal counteranions was investigated in proteoliposomes loaded with a variety of substrates. The intraliposomal concentration of the anions used was 20 mM and the exchange time was 10 min. The date reported in Table II show that 0.1 mM [^{3}H]ATP could be transported against ATP and ADP. Labelled ATP could also be exchanged for GTP and GDP, although the extent of the heterologous ATP/GTP and ATP/GDP exchanges after a 10 min incubation was respectively only 67 and 18% that of the homologous ATP/ATP exchange. In contrast, labelled ATP does not significantly exchange against AMP, cytosine- and uracil-nucleotides, nor substrates of other mitochondrial carriers like citrate, 2-oxoglutarate, malate, phosphate, pyruvate and (not shown) glutamate, glycine, and serine. These results are in agreement with the narrow specificity of the ADP/ATP carrier as characterized in mitochondria (Earnshaw, 1977; Hanson, 1985; Klingenberg, 1985; Wiskich, 1977), and in purified ADP/ATP carrier from corn mitochondria (Genchi *et al.*, 1996).

Table II. Dependence on Internal Substrates of ATP Transport in Reconstituted Liposomes

Internal substrate (20 mM)	ATP transport (nmol 10 min^{-1} mg $protein^{-1}$)
None (CI^- present)	99
ATP	3552
ADP	3260
AMP	270
GTP	2370
GDP	650
CTP	277
CDP	60
UTP	210
Citrate	119
α-Ketoglutarate	112
Malate	188
Phosphate	210
Pyruvate	200

Note. The proteoliposomes were loaded with the indicated substrates. Transport was initiated by adding 0.1 mM [^{3}H]ATP. The results are the means of three experiments.

The sensitivity of the reconstituted ATP/ATP exchange to externally added substrates and inhibitors was also investigated (Tables III and IV). The ATP/ATP exchange was inhibited strongly by ATP and ADP, and less efficiently by GTP and GDP. In contrast, AMP, CTP, CDP, UTP, citrate, α-ketoglutarate, malate, phosphate, and pyruvate had no effect. In addition, ATP/ATP exchange (see Table IV) was inhibited by bongkrekate, which is a known inhibitor of the adenine nucleotide transporter in both plant and animal mitochondria (Earnshaw, 1977; Genchi *et al.*, 1996; Hanson, 1985; Klingenberg, 1985; Vignais, 1976; Wiskich, 1977). In this table it is shown that in tuber mitochondria atractyloside (Passam and Coleman, 1975) and carboxyatractyloside are without effect when added at concentrations normally inhibitory (100 μM) in animal and plant mitochondria. Higher concentrations of atractyloside (5 mM) gave an inhibition of 96%. The same results were obtained with carboxyatractyloside (results not shown; see also Passam and Coleman, 1975). The data reported in Table IV also show that the sulphydryl reagents mersalyl and *p*-hydroxymercuribenzoate (but not *N*-ethylmaleimide), as well as the lysyl-specific reagents phenylisothiocyanate and pyridoxal 5′-phosphate strongly inhibited the reconstituted ATP exchange. In contrast, inhibitors of other mitochondrial metabolite carriers such as 1,2,3-benzenetricarboxylate, phenylglioxale and 2,3-pentanedione (Table IV), phthalonate, and phenylsuccinate (not shown) had no significant effect.

Table III. Sensitivity of ATP/ATP Exchange in Reconstituted Liposomes to Externally Added Substrates

Addition	Inhibition (%)
ATP	98
ADP	90
AMP	11
GTP	69
GDP	23
CTP	11
CDP	6
UTP	5
Citrate	8
α-Ketoglutarate	10
Malate	9
Phosphate	7
Pyruvate	8

Note. The proteoliposomes were loaded with 20 mM ATP and the exchange was started by adding 0.1 mM [^{3}H]ATP. The external substrates were added together with [^{3}H]ATP at a final concentration of 2.0 mM. The control values of uninhibited ATP exchange was 3350 nmol 10 min^{-1} mg $protein^{-1}$. The data are the means of three experiments.

Table IV. Sensitivity of ATP/ATP Exchange in Reconstituted Liposomes to Inhibitors

Addition	Concentration (mM)	Inhibition (%)
Atractyloside	0.1	10
Atractyloside	1.0	54
Atractyloside	2.0	77
Atractyloside	5.0	96
Carboxyatractyloside	0.1	13
Bongkrekate	0.01	93
1,2,3-Benzenetricarboxylate	5.0	9
N-ethylmaleimide	2.0	8
Mersalyl	2.0	97
p-Hydroxymercuribenzoate	2.0	96
Phenylisothiocyanate	10.0	73
Pyridoxal 5′-phosphate	10.0	100
Phenylglioxale	10.0	15
2,3-Pentanedione	10.0	10

Note. The proteoliposomes were loaded with 20 mM ATP and the exchange was started by adding 0.1 mM [^{3}H]ATP. The inhibitors were added together with [^{3}H]ATP except that the SH reagents were added 2 min before the labeled substrate. The control values of uninhibited ATP exchange was 3455 nmol 10 min^{-1} mg $protein^{-1}$. The data are the means of three experiments.

K_m and V_{max} Values of ATP Transport and Inhibition by ADP

To obtain the basic kinetic data of the adenine nucleotide carrier from JATM, the dependence of the

exchange rate on substrate concentration was studied by changing the concentration of externally added [^{3}H]ATP at a constant internal concentration of 20 mM ATP. In nine experiments for the ATP substrate at 25°C an average of 20.4 ± 6.4 μM for the K_m and 0.53 ± 0.11 μmol/min per mg protein for the V_{max} was determined. The inhibition of the reconstituted ATP/ATP exchange by ADP was analyzed in the presence of different substrate concentrations. ADP was identified as competitive inhibitor with respect to ATP. The inhibition constant, K_i, calculated from a double reciprocal plot of the rate of ATP/ATP exchange versus substrate concentrations, was found to be 45.0 ± 3.8 μM for ADP (four experiments).

Partial Protein Sequencing

The purified protein was transferred to a PVDF membrane and sequenced by Edman degradation. The N-terminal sequence of the intact protein was APAEKGLTGF. This partial N-terminal sequence of the first 10 amino acids shows that the first six amino acids and the 10th amino acid are the same as the purified ADP/ATP carrier from maize mitochondria (Genchi *et al.*, 1996).

DISCUSSION

The data presented in this paper demonstrate that we isolated and purified a 33-kDa protein from JATM that catalyzes the transport of ATP, ADP, GTP, and at lower rate GDP. For this purification we used a general scheme applied in our laboratory for the isolation of other mitochondrial carriers involving solubilization with nonionic detergent Triton X-100 in the presence of exogenous cardiolipin and chromatography on hydroxyapatite and Matrex Gel Blue B (Palmieri *et al.*, 1995), with some modification of experimental conditions.

The highly purified nature of the ADP/ATP carrier obtained from Matrex Gel Blue B fraction is demonstrated by the SDS polyacrylamide gel electrophoretic analysis, which indicates the presence of a single mitochondrial band with an apparent molecular mass of 33 kDa. This value falls into the very narrow range of apparent molecular masses between 28 and 34 kDa shown by all mitochondrial metabolite carriers isolated so far both from animal and plant tissues (Palmieri, 1994).

The conclusion that the 33-kDa protein, purified in our laboratory, is in fact the ATP carrier is supported by the following observations: (a) the Matrex Gel Blue B eluate reconstituted in liposomes catalyzes active [^{3}H]ATP/ATP exchange; (b) furthermore, the purified transporter exhibits a substrate specificity (Tables II and III) and an inhibitory sensitivity (Table IV) that partially resembles those observed for the ATP transport system in animal and plant mitochondria (Earnshaw, 1977; Genchi *et al.*, 1996; Hanson, 1985; Klingenberg, 1985; Wiskich, 1977). It is very important to note that the sensitivity of the Jerusalem artichoke carrier to atractyloside (Table IV) and to carboxyatractyloside (results not shown) is different from the other animal and plant carriers. In tuber mitochondria the ATP carrier is quite insensitive to atractyloside; in fact it is only with 5 mM atractyloside we obtain 96% inhibition (Table IV; see also Passam and Coleman, 1975). The same results (not shown) have been obtained with carboxyatractyloside. A sequence of 10 amino acids from the N-terminus have been determined. The first six amino acids and the 10th amino acid of this sequence correspond to the amino acid sequence of the maize adenine nucleotide translocator (Genchi *et al.*, 1996).

It has been reported that mitochondrial carrier protein can fulfill several functions (Engstovà *et al.*, 2000). The ATP carrier is thought to participate in the so-called mitochondrial permeability transition (MTP) (Halestrap *et al.*, 2000, 2002), which is also activated by fatty acid (Schonfeld *et al.*, 1997, 2000). PIC, ANT, UCPI, UCP2, and PUMP have been shown to interact with fatty acids and predicted to mediate the uniport of fatty acid anions. This hallows for the FA uncoupling cycle, suggested by Skulachev (1998) and Engstovà *et al.* (2000). This function might regulate ROS production in plant mitochondria (Moller, 2001) in various physiological conditions, as exposure at chilling temperatures, freezing, wounding or fungi infection. Further studies are necessary to elucidate how the different amino-acid sequences, substrate specificity, and inhibitor sensitivity of ANT protein expressed in various plant and animal tissues permit side functions as uncoupling and consequently defenses to oxidative stress.

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REFERENCES

Bernardi, P., Petronilli, V., Di Lisa, F., and Forte, M. (2001). *Trends Biochem. Sci.* **26**, 112–117.

Bisaccia, F., Indiveri, C., and Palmieri, F. (1985). *Biochim. Biophys. Acta* **810**, 362–369.

Boreky, J., Maia, I. G., Costa, A. D. T., Jezek, P., Chaimovich, H., De Andrade, P. B. M., Vercesi, A. E., and Arruda, P. (2001). *FEBS Lett.* **505**, 240–244.
Brustovetsky, N., and Klingenberg, M. (1996). *Biochemistry* **35**, 8483–8488.
Day, D. A., and Wiskich, J. T. (1984). *Physiol. Veg.* **22**, 241–261.
De Santis, A., Landi, P., and Genchi, G. (1999). *Plant Physiol.* **119**, 743–754.
Douce, R., and Neuburger, M. (1989). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 371–414.
Dulley, I. R., and Grieve, P. A. (1975). *Anal. Biochem.* **64**, 136–141.
Earnshaw, M. J. (1977). *Phytochemistry* **16**, 181–184.
Engstovà, H., Zàckovà, M., Ruzicka, M., Meinhardt, A., Hanus, J., Kramer, R., and Jezek, P. (2000). *J. Biol. Chem.* **276**, 4683–4691.
Genchi, G., De Santis, A., Ponzone, C., and Palmieri, F. (1991). *Plant Physiol.* **96**, 1003–1007.
Genchi, G., Ponzone, C., Bisaccia, F., De Santis, A., Stefanizzi, L., and Palmieri, F. (1996). *Plant Physiol.* **112**, 845–851.
Genchi, G., Spagnoletta, A., De Santis, A., Stefanizzi, L., and Palmieri, F. (1999). *Plant Physiol.* **120**, 841–847.
Halestrap, A. P., Doran, N., Gilles Pie, J. P., and O'Toole, A. (2000). *Biochem. Soc. Trans.* **28**, 170–177.
Halestrap, A. P., McStay, G. P., and Clarke, S. J. (2002). *Biochemie* **84**, 153–166.
Hanson, I. B. (1985). In *Encyclopedia of Plant Physiology, Vol 18: Membrane Transport Systems of Plant Mitochondria* (Douce, R., and Day, D. A., eds.), Springer-Verlag, Berlin, pp. 248–280.
Heldt, H. W., and Flügge, U. I. (1987). In T*he biochemistry of Plants: Subcellular Transport of Metabolites in Plant Cells* (Stumpf, P. K., and Conn, E. E., eds.), Academic Press, New York, pp. 49–85.
Klingenberg, M. (1985). In *The Enzymes of Biological Membranes: The ADP/ATP Carrier in Mitochondrial Membranes, Vol 4.* (Martonosi, A. M., ed.) Plenum, New York, pp. 511–553.
Laemmli, K. (1970). *Nature* **227**, 680–685.
McIntosh, C. A., and Oliver, D. J. (1992). *Plant Physiol.* **100**, 2030–2034.
McIntosh, C. A., and Oliver, D. J. (1994). *Plant Physiol.* **105**, 47–52.
Moller, I. M. (2001). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 561–591.
Morrissey, J. H. (1981). *Anal. Biochem.* **117**, 307–310.
Murai, M., and Yoshida, S. (1998a). *Plant Cell Physiol.* **39**, 87–96.
Murai, M., and Yoshida, S. (1998b). *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, D. A., eds.), Springer-Verlag, New York, pp. 1–24.
Oliver, D. J. (1987). In *Plant Mitochondria: Glycine Uptake by Pea Leaf Mitochondria: A Proposed Model for the Mechanism of Glycine-Serine Exchange* (Moore, A. L., and Beechey, R. B., eds.), Plenum, New York, pp. 219–222.
Palmer, J. M. (1967). *Nature* **216**, 1208–1213.
Palmieri, F., and Klingenberg, M. (1979). *Methods Enzymol.* **56**, 279–301.
Palmieri, F. (1994). *FEBS Lett.* **346**, 48–54.
Palmieri, F., Indiveri, C., Bisaccia, F., and Iacobazzi, V. (1995). *Methods Enzymol.* **260**, 349–369.
Palmieri, L., Rottensteiner, H., Girzalsky, W., Scarcia, P., Palmieri F., and Erdmann, R. (2001). *EMBO J.* **20**, 5049–5059.
Passam, H. C., and Coleman, J. O. D. (1975). *J. Exp. Bot.* **26,** 536–543.
Picault, N., Palmieri, L., Pisano, I., Hodges, M., and Palmieri, F. (2002). *J. Biol. Chem.* **27**, 24204–24211.
Pozueta-Romero, J., Viale, A. M., and Akazawa, T. (1991). *Annu. Rev. Plant Physiol.* **30**, 425–484.
Schonfeld, P., Schluter, T., and Bohnensack, R. (1997). *FEBS Lett.* **420**, 167–170.
Schonfeld, P., Wieckowski, M. R., and Wojtczac, L. (2000). FEBS Lett. **471**, 108–112.
Silva, M. A. P., Moreau, F., Zachowski, A., Mesneau, A., and Roussaux, J. (1999). Plant Sci. **143**, 27–33.
Skulachev, V. P. (1998). *Biochim. Biophys. Acta* **1363**, 100–124.
Takabatake, R., Hata, S., Taniguchi, M., Kouchi, H., Sugiyama, T., and Izui, K. (1999). *Plant Mol. Biol.* **40**, 479–486.
Taniguchi, M., and Sugiyama, T. (1996). *Plant Mol. Biol.* **30**, 51–64.
Taniguchi, M., and Sugiyama, T. (1997). *Plant Physiol.* **114**, 285–293.
Vignais, P. V., Douce, R., Lauquin, G. J. M., and Vignais, P. M. (1976). *Biochim. Biophys. Acta* **440**, 688–696.
Vignais, P. V., Block, M. R., Boulay, F., Brandolin, G., and Lauqin, G. J. M. (1985). In *Structure and Properties of Cell Membranes: Molecular Aspects of Structure-Function Relationships in Mitochondrial Adenine Nucleotide Carrier Vol. 2.* (Bengha, G., ed.) CRC Press, Boca Raton, FL, pp. 139–179.
Vivekananda, J., Beck, C. F., and Oliver, D. J. (1988). *J. Biol. Chem.* **263**, 4782–4788.
Vivekananda, J., and Oliver, D. J. (1989). *Plant Physiol.* **91**, 272–277.
Vivekananda, J., and Oliver, D. J. (1990). *FEBS Lett.* **260**, 217–219,
Wiskich, J. T. (1977). *Annu. Rev. Plant Physiol.* **28**, 45–69.