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Functional reconstitution of *Arabidopsis thaliana* plant uncoupling mitochondrial protein (*At*PUMP1) expressed in *Escherichia coli*

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Abstract The *Arabidopsis thaliana* uncoupling protein (UCP) gene was expressed in *Escherichia coli* and isolated protein reconstituted into liposomes. Linoleic acid-induced H^+ fluxes were sensitive to purine nucleotide inhibition with an apparent K_i (in mM) of 0.8 (GDP), 0.85 (ATP), 0.98 (GTP), and 1.41 (ADP); the inhibition was pH-dependent. Kinetics of *At*-PUMP1-mediated H^+ fluxes were determined for lauric, myristic, palmitic, oleic, linoleic, and linolenic acids. Properties of recombinant *At*PUMP1 indicate that it represents a plant counterpart of animal UCP2 or UCP3. This work brings the functional and genetic approaches together for the first time, providing strong support that *At*PUMP1 is truly an UCP. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Recombinant plant uncoupling protein; Reconstitution in liposome; Fatty acid-induced proton flux; Inhibition by purine nucleotide; *Arabidopsis thaliana*

1. Introduction

The discovery in plants of a purine nucleotide (PN)-sensitive free fatty acids (FAs)-activated uncoupling mitochondrial protein, PUMP [1–3], sharing features similar to uncoupling protein (UCP) 1, raised the possibility of a new mechanism involved in the regulation of thermogenesis and efficiency of oxidative phosphorylation in plants. Since this initial report, the biochemical and physiological properties of PUMP purified from mitochondria of plant tissues have been characterized [4–11], and genes encoding PUMP have been described [12–15].

Similarly to UCP1–3 [16–18], PUMP requires FA for its activity [1,4,5,7,9], either as proton transport cofactors (proton buffering model; [19]) or as a transported substrate (protonophore model). Data from Skulachev [20], Garlid's group [16,21,22], Wojtczak's group [23], and our previous results [5] favor the protonophore model – FA cycling. PUMP has narrower substrate specificity than UCP1 [17,24], it transports hydrophobic anions (FA anions, hexanesulfonate, and undecanesulfonate) but not chloride and pyruvate [4,5]. In potato [4,8,11] and tomato [6,7,9,10] mitochondria, FA removal and the presence of ATP, GTP, or GDP are required for full coupling and, correspondingly, the FA-induced uncoupling is sensitive to PN. Thus, PN can be considered as inhibitory regulators of PUMP, with apparent affinities similar to UCP2 and UCP3 [18]. ATP or GDP inhibition of PUMP-mediated H^+ uniport in potato mitochondria [4] or in proteoliposomes with the reconstituted potato PUMP [5] exhibited an apparent K_i from 0.7 to 0.8 mM. Only 8-azido-ATP has been shown to bind to potato PUMP with a K_d of 21 μM in the dark [25]; no binding of natural PN has been reported.

PUMP may be ubiquitously present in plants. Polyclonal anti-potato PUMP antibodies detected the presence of a ~32 kDa band in various fruits, including those with a climacteric respiratory rise [26]. Full-length cDNAs encoding PUMP have been isolated from potato [12], *Arabidopsis thaliana* [13,15], and thermogenic plant skunk cabbage [14]. The potato cDNA (*St*UCP) most likely encodes the PUMP protein purified from potato tubers [4], since matrix-assisted laser desorption ionization-mass spectroscopy analysis of peptides resulting from trypsin cleavage of PUMP yielded 35% coverage of *St*UCP sequence (Ružicka, M., Novák, P., Žácková, M., Costa, A.D.T., Vercesi, A.E. and Ježek, P., unpublished).

In this report, by expressing a full-length cDNA encoding the *Arabidopsis* UCP *At*PUMP1 [13] in *Escherichia coli*, we unequivocally demonstrate the existence of a mitochondrial UCP in plants. In addition, we establish an expression system suitable for study of structure/function relationships of PUMP. The results obtained with reconstituted recombinant *At*PUMP1 demonstrate that it has properties similar to reconstituted recombinant UCP2 or UCP3. Therefore, the uncou-

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Abbreviations: CSPD, disodium 3-(4-methoxyspiro{1,2-dioethane-3,2′-(5′-chloro) tricyclo[3.3.1.1^{3,7}]decane}-4-yl)phenylphosphate; decylPOE, decylpolyoxyethylene; DTT, dithiothreitol; EM, external medium; FA, fatty acid; IM, internal medium; IPTG, isopropyl-β-D-thiogalactopyranoside; PN, purine nucleotides; PUMP, plant uncoupling mitochondrial protein; SLS, sodium lauroylsarcosinate; UCP1, brown adipose tissue uncoupling protein; UCP2, ubiquitous uncoupling protein; UCP3, skeletal muscle- and brown adipose tissue-specific uncoupling protein; UCP4, brain-specific uncoupling protein; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; TEA, tetraethyl ammonium; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid

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pling phenomenon in plants can be assigned directly and definitively to a specific gene and its protein product that represents the plant counterpart of the UCP2 or UCP3.

2. Materials and methods

2.1. Materials

FAs, nucleotides, and other chemicals were from Sigma, undecanesulfonate from Lancaster (UK), and Bio-Beads SM2 from Bio-Rad. SPQ (6-methoxy-*N*-(3-sulfopropyl)quinolinium) was from Molecular Probes. Hybond N membranes and Hyperfilms MP were from Amersham and the CSPD (disodium 3-(4-methoxyspiro{1,2-dioethane-3,2′-(5′-chloro) tricyclo[3.3.1.1^{3,7}]decane}-4-yl)phenylphosphate) was from Tropix.

2.2. Construction of the *At*PUMP1 expression vector

Standard cloning procedures [27] were used for construction of *At*PUMP1 encoding plasmid. The *At*PUMP1 open reading frame was obtained by PCR from a full-length cDNA clone [13] using *Pfu* DNA polymerase and gene-specific primers supplemented with restriction sites for *Nco*I and *Hin*dIII. Gel-purified PCR fragment was digested by *Hin*dIII, blunted, cut with *Nco*I, and cloned into the *Nco*I/*Bam*HI-blunt restrict of vector pET-3d (Novagen). The construct was sequenced (ABI 310 automatic sequencer, Perkin-Elmer). *E. coli* BL21(DE3) pLysS (T7 expression system, Stratagene) was transformed by the vector.

2.3. Expression of *At*PUMP1 and isolation of inclusion bodies

Transformed *E. coli* cells were grown overnight at 37°C in Luria–Bertani medium supplemented by 100 mg l^{-1} ampicillin and 25 mg l^{-1} chloramphenicol. The culture was diluted 1:100 in the same medium and grown at 37°C to the mid-log phase ($OD_{600}\sim0.5$), and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. Harvested cells were lysed in 100 mM Tris–HCl, pH 7.0, 5 mM EDTA, and 5 mM dithiothreitol (DTT) (6 freeze–thaw cycles – liquid nitrogen/37°C). After breaking the genomic DNA by sonication (6×30 s), the suspension was centrifuged (20 000×*g*, 30 min). Pellets of inclusion bodies were washed twice in 1% Triton X-100 and 1 M urea and the denaturants were washed out in 50 mM Tris–HCl, pH 7.0, 1 mM EDTA, and 2 mM DTT.

2.4. Reconstitution of *At*PUMP1 and fluorescent monitoring of H^+ fluxes

4 mg of inclusion bodies were washed twice in 1 ml of internal medium (IM; 28.8 mM tetraethyl ammonium (TEA)–*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) with 9.2 mM TEA, pH 7.2, 84.4 mM TEA_2SO_4 and 0.6 mM TEA–EGTA). The pellet was pre-solubilized in 1 ml of IM containing 0.3% sodium lauroylsarcosinate (SLS) and centrifuged (13 000×*g*, 10 min), and solubilized in 1 ml of IM containing 1.67% SLS and 1% decylpolyoxyethylene (decylPOE) at 4°C for 2.5 h. The insoluble fraction was removed by centrifugation. This procedure yielded a crude preparation (with a negligible contamination by inclusion body proteins) used for reconstitutions.

Lipid film was prepared from ethyl ether solution of 39 mg egg yolk lecithin, 1.66 mg cardiolipin, and 0.66 mg phosphatidic acid by a stream of nitrogen. The film was hydrated in 975 μl of IM by vortexing at 50°C under nitrogen and solubilized by adding 76 mg of decylPOE. The micelles were cooled down on ice and mixed with 2 mM SPQ probe and ~50 μg of solubilized *At*PUMP1 (lipid/protein ratio of 830:1) and incubated in a column of Bio-Beads SM2 for 2.5 h at 4°C. Proteoliposomes were recovered by centrifugation. Traces of detergent were removed by reincubation of proteoliposomes with fresh Bio-Beads for 10 min and recentrifugation. External SPQ was removed by gel filtration through a Sephadex G25-300 spin column. All columns were pre-washed with IM.

H^+ flux assays were based on the quenching of SPQ fluorescence by TES^- [28]. Vesicles (35 μl; 1.4 mg lipids) were added to 1.5 ml of external medium (EM; 28.8 mM TEA–TES (9.2 mM TEA), pH 7.2, 84.4 mM K_2SO_4, and 0.6 mM TEA–EGTA). FAs were added in 10 s and, 20 s later, a H^+ efflux was initiated by 1.3 μM valinomycin. The fluorescence was calibrated to $[H^+]$ by the addition of 2 M KOH aliquots in the presence of 1.5 μM nigericin to proteoliposomes suspended in IM. To convert fluorescent data into 'H^+ traces', fluorescence data were fitted by a modified Stern–Volmer equation,

$$[H^+] = (1/K_q)(F_0-F)/(F-L) \quad (1)$$

where F is the experimental and F_0 the unquenched fluorescence. Parameters K_q (quenching constant) and L (background, mostly light scattering) were obtained by linear regression of F vs. (F_0-F)/[KOH] plot. Corresponding rates (in mM s^{-1}) were derived from 'H^+ traces', multiplied by the internal proteoliposome volume (V, estimated from volume distribution of the SPQ [16]), and corrected for protein content to yield final rates in amol H^+ s^{-1} (mg protein)$^{-1}$. When expressed per nmol of PUMP dimer, rates represented the minimum values for the turnover number, since not all of the protein was inserted into the vesicles. To compare the results from vesicles of different volumes, the rates were expressed as flux densities in pmol H^+ s^{-1} μm^{-2}, total vesicle surface being derived from internal vesicle volume.

2.5. Preparation of polyclonal antibodies against recombinant *At*PUMP1

Inclusion bodies were extracted with 8 M guanidine–HCl and the insoluble fraction was removed by centrifugation (20 000×*g*; 30 min). The protein was diluted to 1–2 mg ml^{-1} with 4 M guanidine–HCl and refolded via denaturant removal by slow pumping (2.2 ml h^{-1}) of the solution into a 'folding buffer' (50 mM Tris–HCl, pH 7.0, 1 mM EDTA, 2 mM DTT, 2% SLS, and 1% decylPOE). The protein was concentrated by acetone precipitation and resolubilized in folding buffer. Aliquots of *At*PUMP1 (600 μg) were electrophoresed and the protein band, detected with ice cold 150 mM KCl, was cut out, homogenized in incomplete Freud's adjuvant, and injected subcutaneously into each of two rabbits. Injection was repeated after 16 days. The rabbits were bled 21 days later and crude sera obtained by standard procedures.

2.6. Polyacrylamide gel electrophoresis (PAGE) and Western blot

Proteins were separated on a pair of 10% polyacrylamide gels (standard sodium dodecyl sulfate (SDS)–PAGE). One gel was stained with 0.27% (w/v) Coomassie brilliant blue R-250. Protein bands from the other gel were transferred to a nylon membrane (Hybond N) in a semi-dry blotting apparatus (Pharmacia). The membrane was blocked overnight in 20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20, and 10% (w/v) non-fat dry milk and incubated with the anti-*At*PUMP1 or anti-potato PUMP [26] polyclonal antibodies (1:1000 dilution). After incubation with anti-rabbit IgG alkaline phosphatase conjugate (1:5000 dilution), the membrane was developed in the dark in 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and CSPD (1:2000 dilution) for 5 min. The bands were visualized by autoradiography and scanned with an Eagle-Eye photo documentation system.

3. Results

3.1. Characterization of the expression product

Expression of *At*PUMP1 in *E. coli* produced an additional polypeptide with an apparent molecular mass of 30 kDa in cell lysates of induced bacteria (Fig. 1A, lane Bi vs. Bn) that was also present in the inclusion body fraction (Fig. 1A, lane IB). This band was apparent after pre-solubilization of the inclusion bodies with 0.3% SLS (Fig. 1A, lane S). Subsequent addition of decylPOE, accompanied by a refolding process, yielded a ~32 kDa protein (Fig. 1A, lane PUMP), corresponding to the molecular mass for PUMP (32.5 kDa) estimated from the cDNA sequence. Immunoblot analysis indicated that both the anti-*At*PUMP1 antibody and the anti-potato PUMP antibody [9,11,26] recognized the overexpressed 32 kDa protein (Fig. 1B,C), confirming the identification of the recombinant protein as *At*PUMP1.

3.2. FA-induced H^+ efflux in proteoliposomes containing recombinant *At*PUMP1

Fig. 2 illustrates the H^+ fluxes in proteoliposomes containing reconstituted recombinant *At*PUMP1. Acidification of the

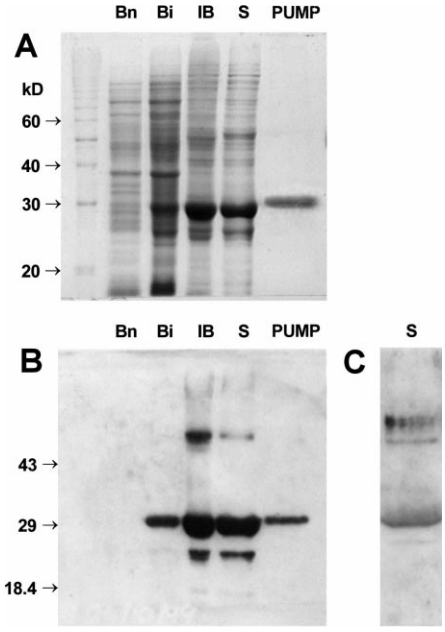

Fig. 1. Characterization of recombinant *At*PUMP1 expressed in *E. coli*. A: SDS–PAGE stained with Coomassie blue. The lanes correspond to the molecular mass standards (first lane, numbers to the left indicate molecular mass in kDa), extracts of non-induced (lane Bn) or IPTG-induced (lane Bi) bacteria expressing *At*PUMP1, SDS-solubilized inclusion bodies (lane IB), inclusion bodies pre-solubilized with 0.3% SLS (lane S) and the solubilized *At*PUMP1 used for reconstitution (lane PUMP). B and C: Western blots showing positive reaction of polyclonal antibodies anti-*At*PUMP1 and anti-potato PUMP, respectively (lane names are equal to A).

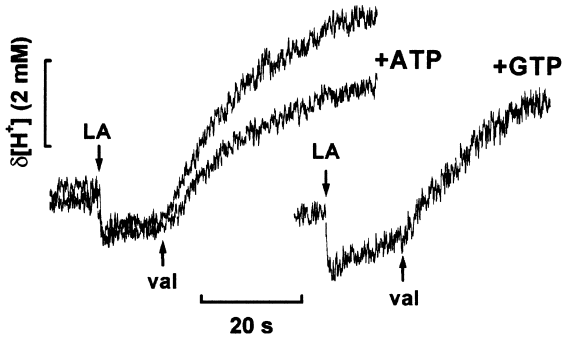

Fig. 2. Typical H^+ fluxes in proteoliposomes with the reconstituted recombinant *At*PUMP1. The H^+ traces are shown for a H^+ efflux in proteoliposomes containing *At*PUMP1 induced by 26.6 μM linoleic acid in the presence of 1.3 μM valinomycin at approximately 179.5 mV (165 mM [K^+] outside, 0.168 mM [K^+] inside) and in the absence or presence of 4 mM ATP (left traces) or GTP (right trace). The H^+ flux in the control was 0.16 nmol H^+ s^{-1} (mg lipid)$^{-1}$ (0.13 when the basal rate with no linoleic acid was subtracted), which corresponded to 8.1 μmol H^+ min^{-1} (mg total protein)$^{-1}$.

vesicles occurred after addition of 26.6 μM linoleic acid, indicating the redistribution of FA molecules in both leaflets of the lipid bilayer leading to internal H^+ release. The addition of 1.3 μM valinomycin produced a H^+ efflux that represented FA cycling [5,10,16,18,21,22]. At ~180 mV, the H^+ efflux induced by 26.6 μM linoleic acid amounted to 0.16 nmol H^+ s^{-1} (mg lipid)$^{-1}$ (0.13 when basal rates at zero linoleic acid concentration were subtracted). This corresponded to 8.1 μmol H^+ min^{-1} (mg total protein)$^{-1}$ and a minimum turnover number of 8.75 s^{-1} for dimeric PUMP. Comparable values were obtained with PUMP isolated from potato mitochondria and the corresponding dose of linoleic acid [5]. Sodium undecanesulfonate (0.5 mM) completely blocked the linoleic acid-induced H^+ flux (data not shown), as demonstrated for UCP1 [16], UCP2, UCP3 [18], and potato PUMP [5]. In contrast, atractylate (cf. [23]) or $MgSO_4$ (up to 7 mM) had no effect.

3.3. Kinetics of FA-induced AtPUMP1-mediated H^+ efflux

The kinetics of FA cycling mediated by *At*PUMP1 were evaluated by varying the total FA concentration. Protein-independent basal H^+ fluxes were simulated in protein-free liposomes, under identical FA and valinomycin concentrations and membrane potential. Data, corrected for the different (lower) volume of proteoliposomes, were used for a theoretical estimation of the basal H^+ flux in proteoliposomes. The apparent H^+ permeability and H^+ flux density of protein-free vesicles were evaluated in the presence of 1.3 μM valinomycin and increasing concentrations of palmitic, linoleic, and lauric acids (Fig. 3A) and myristic, oleic, and linolenic acids (Fig. 3C) up to 533 μM. The basal H^+ efflux increased linearly with FA concentrations and did not exceed 0.2, 0.28 or 0.32 nmol H^+ s^{-1} (mg lipid)$^{-1}$ (Fig. 3A) or 0.03, 0.28, and 0.44 nmol H^+ s^{-1} (mg lipid)$^{-1}$ (Fig. 3C), respectively, which corresponded to H^+ efflux densities of 27×10^{-6}, 38×10^{-6} and 43×10^{-6} amol H^+ s^{-1} μm^{-2} (Fig. 3A) and of 41, 60, and 92 amol H^+ s^{-1} μm^{-2} (Fig. 3C), respectively. FAs with higher partition coefficients (3.75×10^5, 3.6×10^5, 10^5, 5×10^4 and 5×10^3 for palmitic, oleic, linoleic, linolenic, and lauric acids, respectively) [29,30] induced lower basal H^+ fluxes. At 33 μM palmitic, linoleic, and lauric acid, these fluxes were close to the membrane H^+ permeability without FA and amounted to 0.046, 0.056 and 0.04 nmol H^+ s^{-1} (mg lipid)$^{-1}$ which corresponded to 6, 7.7, and 5.5 amol H^+ s^{-1} μm^{-2}, respectively. At 33 μM myristic, oleic, and linolenic acids similar rates were measured – 0.008, 0.044, and 0.051, respectively (11, 9, and 11 amol H^+ s^{-1} μm^{-2}, respectively). The measured proteoliposomal H^+ flux density without FA was slightly higher (7–11 amol H^+ s^{-1} μm^{-2}).

Evaluation of the kinetics of linoleic acid cycling mediated by *At*PUMP1 produced a typical dose response (Fig. 3A) that exceeded the H^+ fluxes in liposomes. The corresponding Eadie–Hofstee plot yielded a K_m of 43 μM when using an iterative procedure for evaluating the proteoliposomal basal H^+ flux (Fig. 3B) giving a V_{max} of 0.32 nmol H^+ s^{-1} (mg lipid)$^{-1}$ that corresponded to minimum turnover numbers of 16.5 s^{-1}

Table 1
K_m and V_{max} values for FA-dependent, *At*PUMP-mediated proton flux

Constant	FA					
	Linoleic	Lauric	Palmitic	Myristic	Oleic	Linolenic
K_m (μM)	43	55	129	44	92	130
V_{max} (nmol H^+ s^{-1} (mg lipid)$^{-1}$)	0.32	0.22	0.43	0.24	0.73	0.84

for dimeric PUMP. All evaluations using Eadie–Hofstee plots for linoleic, lauric, and palmitic acidd (Fig. 3B), and myristic, oleic, and linolenic acids (Fig. 3D) are summarized in Table 1.

3.4. Inhibition of FA-induced *At*PUMP1-mediated H^+ efflux by PN

PN inhibited up to 50% of the observed H^+ efflux induced by linoleic acid in proteoliposomes containing *At*PUMP1 (Fig. 2). The incomplete inhibition most probably resulted from the even or random orientation of *At*PUMP1 molecules bearing the binding site [5,10,16,22]. When this was accounted for, it was possible to construct dose–response curves (Fig. 4A–C) for the inhibitory effect of GDP (K_i of 0.8 mM at pH 7.1), GTP (K_i of 0.98 mM at pH 7.1), ATP (K_i of 0.85 mM at pH 7.1), and ADP (K_i of 1.41 at pH 7.1; Fig. 4C). The

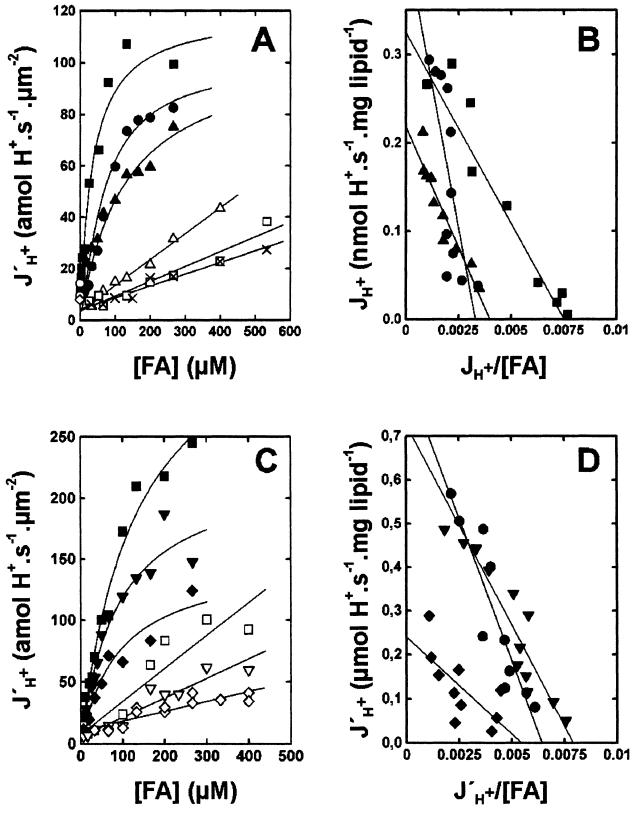

Fig. 3. Kinetics of FA-induced H^+ fluxes in liposomes and proteoliposomes with the reconstituted recombinant *At*PUMP1. A and C show comparison of H^+ flux densities in protein-free liposomes (× and open symbols) and proteoliposomes (solid symbols). The apparent H^+ flux density of protein-free vesicles in the presence of: palmitic (×), linoleic (□), and lauric (△) acids in A, and myristic (◇), oleic (□), and linolenic acids (▽) in C is compared with densities of *At*PUMP1-mediated H^+ fluxes induced by linoleic (■), palmitic (●), and lauric (▲) acids in A, and myristic (◆), oleic (■), and linolenic acids (▼) in C. In A are shown basal H^+ flux densities (no FAs) in protein-free liposomes (◇) and proteoliposomes (○). Measurements were conducted in the presence of 1.3 μM valinomycin. The fluxes were calculated per μm^2 of the vesicle membrane. B and D show Eadie–Hofstee plots for the kinetics of FA cycling with the kinetic data for H^+ fluxes induced by linoleic (■), palmitic (●), and lauric (▲) acids (B), and myristic (◆), oleic (■), and linolenic (▼) acids (D) in proteoliposomes with *At*PUMP1. Basal H^+ fluxes were not subtracted, except linoleic acid, where subtracted basal flux J_B was obtained iteratively, reaching linearization of the plot in the low concentrations (iterated J_B was slightly lower than the measured J_B, cf. panel A). The derived K_m and V_{max} are summarized in Table 1.

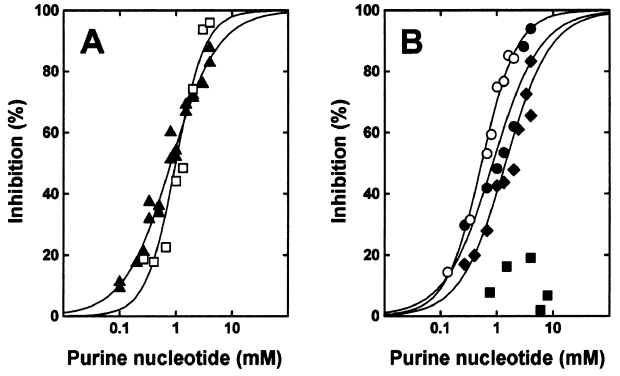

Fig. 4. Dose–response curves for the inhibition of FA-induced *At*PUMP1-mediated H^+ fluxes by GDP (▲) and GTP (□) at pH 7.1 in A, or by ADP (◆), and ATP at pH 6.3 (○), 7.1 (●), and 7.8–8 (■) in B. The dose–response curves (solid lines) represented inhibition of the outwardly oriented PN binding sites (~50%). The derived K_i were 0.8 mM (GDP, two experiments), 0.98 mM (GTP), 1.41 mM (ADP), 0.85 mM (ATP at pH 7.1), and 0.54 (ATP at pH 6.3), with Hill coefficients of 1, except for GTP ($n = 1.7$) and ATP at pH 6.3 ($n = 1.4$). TEA–TES in EM of pH 8 (7.8 at high ATP concentrations) was replaced by Tris–TES and EM of pH 6.3 contained 14.4 mM TES and 14.4 mM 2-[*N*-morpholino]ethanesulfonic acid (9.2 mM TEA^+).

alkaline external pH diminished the inhibitory effect of ATP, so that inhibition did not exceed 20% at pH from 8.0 to 8.5 (Fig. 4B). In contrast, ATP inhibition was stronger at pH 6.3 with a K_i of 0.54 mM (Fig. 4B).

4. Discussion

The results presented in this work provide evidence that *At*PUMP1 expressed in an artificial prokaryote system exhibited functional features of UCP2 and UCP3. Millimolar levels of ATP, ADP, GDP, or GTP blocked the FA-mediated protonophoric function of *At*PUMP1 leading to uncoupling and the K_i values for this inhibition were very close to those previously found for PUMP isolated from tomato mitochondria [10]. Moreover, we show for the first time that alkaline pH decreases the inhibitory effect of PN on PUMP, with inhibition by ATP increasing slightly at pH 6.3. Based on these results, we can predict that the PN binding site in *At*PUMP1 has similar properties to that of UCPs but with lower affinity, as observed for mammalian UCP2 and UCP3 [18]. In contrast, UCP1 has a much higher affinity for PN. Putative insensitivity of UCP2 and UCP3 to GDP was found in physiological studies of the residual proton leak in mitochondrial preparations from brown adipose tissue of UCP1-deficient mice [31]. However, all experiments were done in the presence of 0.5% bovine serum albumin, i.e. in the absence of free FA that is normally required for full activity of UCPs. Moreover, the blockage by 1 mM GDP is complete in the case of UCP1 (K_i ~20 μM) but not for UCP2 and UCP3 (K_i ~1 and ~1.2 mM, respectively) [18]. Thus, these experiments cannot prove that UCP2 and UCP3 are GDP-insensitive. Substitution of Phe-267 by Tyr in the supposed PN binding site of UCP1 led to a decrease of its affinity to GDP [32]. However, UCP2–4 and *At*PUMP1–2 have a tyrosine at this position [33,34]. Nevertheless, all UCPs have the residues that were previously indicated to be essential for PN binding to UCP1 [34].

The H^+ efflux density induced by various FAs in proteoliposomes containing *At*PUMP1 exceeded the basal H^+ efflux density in the presence of FA in protein-free liposomes, and hence the excess flux must be ascribed to the function of *At*PUMP1. The maximum rates for proteoliposomes with *At*-PUMP1 in the presence of linoleic acid were similar to those measured with the isolated native potato PUMP [5]. *At*-PUMP1 does not discriminate significantly among FA, since the FA tested interacted with *At*PUMP1 to a similar extent, i.e. they exhibited K_m values in the range between 40 and 130 μM and V_{max} between 0.2 and 0.84 μmol H^+ s^{-1} (mg lipid)$^{-1}$. For linoleic, myristic, and lauric acids, the total apparent K_m values were very similar (43 μM, 44 μM, and 55 μM), in spite of the different partition coefficients (10^5, 10^5, and 5×10^3, respectively). This accounts for membrane concentrations of 61 mM, 62 mM and 65 mM (equation 1 in [30]). Palmitic acid (the highest partition coefficient of 3.75×10^5) and linolenic acid (with a quite low partition coefficient of 5×10^4) showed a higher K_m of 129 (or 130) μM, corresponding to 183 mM in the membrane for palmitic and 181 mM for linolenic acid. Finally, oleic acid exhibited an intermediate K_m of 92 μM (131 mM in the membrane). The V_{max} values derived from the differential rates were higher either for FA with higher partition coefficients (palmitic, oleic and linoleic acids) or for polyunsaturated FA such as linolenic acid.

The functional reconstitution of recombinant mitochondrial carrier proteins is a useful tool for ascribing functional properties to a gene and the only way to study structure/function relationships in proteins by site-directed mutagenesis. This approach has already been used in the case of UCP2 and UCP3 that have not yet been isolated from native tissues [18]. The results reported in this report evidenced that the recombinant *At*PUMP protein expressed in *E. coli* has properties similar to those of the isolated native PUMP and have provided a definitive proof that *At*PUMP1 is an UCP.

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References

[1] Vercesi, A.E., Martins, I.S., Silva, M.A.P., Leite, H.M.F., Cuccovia, I.M. and Chaimovich, H. (1995) Nature 375, 24.
[2] Vercesi, A.E., Chaimovich, H. and Cuccovia, I.M. (1997) Rec. Res. Dev. Plant Physiol. 1, 85–91.
[3] Vercesi, A.E., Ježek, P., Costa, A.D.T., Kowaltowski, A.J., Maia, I.G. and Arruda, P. (1998) in: Plant Mitochondria, From Gene to Function (Moller, I.M., Gardeström, P., Glimelius, K. and Glaser, E., Eds.), pp. 435–440, Backhuys Publishers, Leiden.
[4] Ježek, P., Costa, A.D.T. and Vercesi, A.E. (1996) J. Biol. Chem. 271, 32743–32748.
[5] Ježek, P., Costa, A.D.T. and Vercesi, A.E. (1997) J. Biol. Chem. 272, 24272–24278.
[6] Jarmuszkiewicz, W., Almeida, A.M., Sluse-Goffart, C., Sluse, F.E. and Vercesi, A.E. (1998) J. Biol. Chem. 273, 34882–34886.
[7] Sluse, F.E., Almeida, A.M., Jarmuszkiewicz, W. and Vercesi, A.E. (1998) FEBS Lett. 433, 237–240.
[8] Kowaltowski, A.J., Costa, A.D.T. and Vercesi, A.E. (1998) FEBS Lett. 425, 213–216.
[9] Almeida, A.M., Jarmuszkiewicz, W., Khomsi, H., Arruda, P., Vercesi, A.E. and Sluse, F.E. (1999) Plant Physiol. 119, 1323–1329.
[10] Costa, A.D.T., Nantes, I.L., Ježek, P., Leite, A., Arruda, P. and Vercesi, A.E. (1999) J. Bioenerg. Biomembr. 31, 527–533.
[11] Nantes, I.L., Fagian, M.M., Catisti, R., Arruda, P., Maia, I.G. and Vercesi, A.E. (1999) FEBS Lett. 457, 103–106.
[12] Laloi, M., Klein, M., Reismeier, J.W., Müller-Röber, B., Fleury, C., Bouillaud, F. and Ricquier, D. (1997) Nature 389, 135–136.
[13] Maia, I.G., Benedetti, C.E., Leite, A., Turcinelli, S.R., Vercesi, A.E. and Arruda, P. (1998) FEBS Lett. 429, 403–406.
[14] Ito, K. (1999) Plant Sci. 149, 167–173.
[15] Watanabe, A., Nakazono, M., Tsutsumi, N. and Hirai, A. (1999) Plant Cell. Physiol. 40, 1160–1166.
[16] Garlid, K.D., Orosz, D.E., Modriansky, M., Vassanelli, S. and Ježek, P. (1996) J. Biol. Chem. 271, 2615–2620.
[17] Ježek, P. and Garlid, K.D. (1998) Int. J. Biochem. Cell Biol. 30, 1163–1168.
[18] Jaburek, M., Varecha, M., Gimeno, R.E., Dembski, M., Ježek, P., Zhang, M., Burn, P., Tartaglia, L.A. and Garlid, K.D. (1999) J. Biol. Chem. 274, 26003–26007.
[19] Klingenberg, M. (1990) Trends Biochem. Sci. 15, 108–112.
[20] Skulachev, V.P. (1991) FEBS Lett. 294, 158–162.
[21] Ježek, P., Modrianský, M. and Garlid, K.D. (1997) FEBS Lett. 408, 161–165.
[22] Ježek, P., Modrianský, M. and Garlid, K.D. (1997) FEBS Lett. 408, 166–170.
[23] Wojtczak, L., Wieckowski, M.R. and Schönfeld, P. (1998) Arch. Biochem. Biophys. 357, 76–84.
[24] Ježek, P. and Borecký, J. (1998) Am. J. Physiol. 275, C496–C504.
[25] Saviani, E.E., Da Silva Jr., A. and Martins, I.S. (1997) Plant Physiol. Biochem. 35, 701–706.
[26] Ježek, P., Engstová, H., Žácková, M., Vercesi, A.E., Costa, A.D.T. and Arruda, P. (1998) Biochim. Biophys. Acta 1365, 319–327.
[27] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[28] Orosz, D.E. and Garlid, K.D. (1993) Anal. Biochem. 210, 7–15.
[29] Richieri, G.V., Ogata, R.T. and Kleinfeld, A.M. (1995) J. Biol. Chem. 270, 15076–15084.
[30] Garlid, K.D., Jaburek, M. and Ježek, P. (1998) FEBS Lett. 438, 10–14.
[31] Monemdjou, S., Kozak, L.P. and Harper, M.-E. (1999) Am. J. Physiol. 276, E1073–E1082.
[32] Bouillaud, F., Arechaga, I., Petit, P.X., Raimbault, S., Levi-Meyrueis, C., Casteilla, L., Laurent, M., Rial, E. and Ricquier, D. (1994) EMBO J. 13, 1990–1997.
[33] Mao, W., Yu, X.X., Zhong, A., Li, W., Brush, J., Sherwood, S.W., Adams, S.H. and Pan, G. (1999) FEBS Lett. 443, 326–330.
[34] Ježek, P. and Urbánková, E. (2000) IUBMB Life 49, 63–70.