Low temperature and aging-promoted expression of PUMP in potato tuber mitochondria

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Abstract In this communication, we show that the plant uncoupling mitochondrial protein (PUMP) present in potato tuber mitochondria is induced by aging at 28°C and that this induction is strongly stimulated when the potato tubers are stored at low temperature (4°C). PUMP activity was detected by the degree of linoleic acid (LA)-induced ATP-sensitive mitochondrial uncoupling measured as a function of the decrease in membrane potential ($\Delta\Psi$). The PUMP content was evaluated by immunoblot analysis using polyclonal antibodies raised against potato PUMP that specifically detected a 32 kDa band. In agreement with the effect of LA on $\Delta\Psi$, the content of the 32 kDa band increased during storage and was stimulated by low temperature. These results support the proposed role of PUMP in plant thermogenesis and possibly in fruit ripening and senescence.

Key words: Plant uncoupling mitochondrial protein; Thermogenesis; Potato tuber mitochondrion; Reactive oxygen

1. Introduction

The brown adipose tissue mitochondria (BATM) in newborn, hibernating, or cold-adapted mammals possess a 32 kDa uncoupling protein (UCP1) [1] that, in the presence of fatty acids (FA), allows the protons extruded by the respiratory chain to re-enter the matrix and bypass the ATP synthase, thus promoting dissipation of $\Delta\mu H^+$ as heat, in a process called non-shivering thermogenesis. The UCP1 content of BATM increases by an order of magnitude during cold stress [2]. Two other mitochondrial uncoupling proteins have recently been described. UCP2 is ubiquitously found in many tissues and thought to play a role in diseases such as diabetes and obesity, and in responses to infection [3], whereas UCP3, found exclusively in skeletal muscle mitochondria, is probably involved in thermogenesis [4].

UCP1 may mediate uncoupling via a FA cycling mechanism [5,6] in which UCP1 translocates FA anions outwards. Subsequently, FA anions become protonated and enter mitochondria in this form, leaving the proton in the matrix [6]. A UCP1-like uncoupling protein was recently discovered in potato tuber mitochondria and named PUMP (plant uncoupling mitochondrial protein) [7]. Using antibodies raised against potato PUMP, this protein has also been detected in various fruits [8] and evidence for its possible role in respiratory energy dissipation during tomato fruit ripening has also been provided [9]. Similarly to UCP1, PUMP has been characterized as a hydrophobic protein that is not retained on hydroxyapatite in a detergent micellar solution [7,10,11]. PUMP is also weakly inhibited by purine nucleotides and translocates FA anions [10–13]. The expression of the StUCP gene, which encodes an uncoupling protein in potato [14], and the At-PUMP gene, which encodes PUMP in *Arabidopsis thaliana* [15], is stimulated by low temperature. Here we have analyzed both the activity and content of PUMP during aging of potato tubers stored at 28°C or 4°C.

2. Materials and methods

2.1. Isolation of potato tuber mitochondria and incubation procedure

Mitochondria were isolated by conventional differential centrifugation, as previously described [16]. The potatoes (*Solanum tuberosum* L., cv. 'bintje') were obtained from a commercial source 3 days after harvesting (referred to here as fresh potatoes). The mitochondria were incubated in a reaction medium (30°C) containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer, pH 7.2, 0.33 mM EGTA, 1.0 mM $MgCl_2$, 2.5 mM inorganic phosphate, and 5 mM potassium succinate. Where indicated, 1.2 μg oligomycin/mg protein, 300 μM propranolol, and 7.0 μM atractyloside were also present. The results are representative of at least three experiments.

2.2. Measurements of mitochondrial transmembrane electrical potential ($\Delta\Psi$)

Mitochondrial $\Delta\Psi$ was estimated by the extent of safranin O fluorescence decrease, recorded on a Hitachi F-4010 fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 495 nm and 586 nm, with a slit width of 5 nm.

2.3. Antibody preparation

Purified PUMP was separated by SDS-PAGE on 10% gels. After electrophoresis [17], the gel was washed three times with cold distilled water for 10 min and the protein stained with cold 250 mM KCl. The protein band containing purified PUMP was excised from the gel, crushed, and mixed (1:1) with incomplete Freund's adjuvant. Four milliliters of the mixture, corresponding to ~300 μmol protein, was used to immunize a rabbit which subsequently received booster injections 4 weeks apart. The rabbit was bled 2 weeks after the last injection. After removal of the clot, the serum was divided into aliquots and stored at −20°C.

2.4. Immunoblotting

Immunoblotting was performed as described by Timmons and Dunbar [18]. After electrophoresis, the gel was soaked for 10 min in 25 mM Tris base containing 190 mM glycine and 20% methanol. The

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Abbreviations: $\Delta\Psi$, mitochondrial transmembrane electrical potential; $\Delta\mu H^+$, proton electrochemical gradient; BATM, brown adipose tissue mitochondria; BSA, bovine serum albumin; FA, fatty acids; LA, linoleic acid; PM, potato mitochondria; PUMP, plant uncoupling mitochondrial protein; UCP, uncoupling proteins

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proteins were electrotransferred to nylon membranes (Hybond N, Amersham) in a semidry blotting apparatus (Pharmacia) after which the membranes were blocked overnight at 4°C in 20 mM Tris, pH 7.4, 137 mM NaCl, 0.1% Tween 20, and 5% non-fat dry milk. The blocked membranes were washed three times in the same buffer without milk, followed by incubation with an anti-rabbit IgG alkaline phosphatase conjugate. Finally, the membranes were incubated for 20 min in the dark in a developing mixture containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 1:1000 solution of CSPD (Tropix). The bands were detected by autoradiography.

2.5. Chemicals

Most of the chemicals, including atractyloside, oligomycin, safranin O, linoleic acid, ATP, succinate, propranolol, and HEPES were purchased from Sigma (St. Louis, MO, USA). The materials for immunoblots (Hybond N membranes, Hyperfilms MP) were purchased from Amersham, and the chemiluminescent substrate for autoradiography (CSPD) was from Tropix (MA, USA). All other reagents were commercial products of the highest purity available.

3. Results and discussion

Fig. 1 shows that the transmembrane electrical potential, estimated by the extent of safranin fluorescence decrease, was smaller in mitochondria isolated in the absence of bovine serum albumin (BSA), from potatoes stored at 4°C for 7 days (line a) than that observed for potatoes stored at 28°C (line b) for the same period of time. However, the consecutive addition of ATP and BSA to both preparations produced larger coupling effects in mitochondria from potatoes stored at low temperature, thus equalizing $\Delta\Psi$ in both preparations. This was also shown by the subsequent elimination of $\Delta\Psi$ by KCN. The larger coupling effects of ATP and BSA in line a may be interpreted in terms of a higher content of contaminant-free FA (the PUMP substrates) or a higher content of PUMP in potato tubers stored at low temperature or both. To examine these possibilities, we investigated the effect of LA

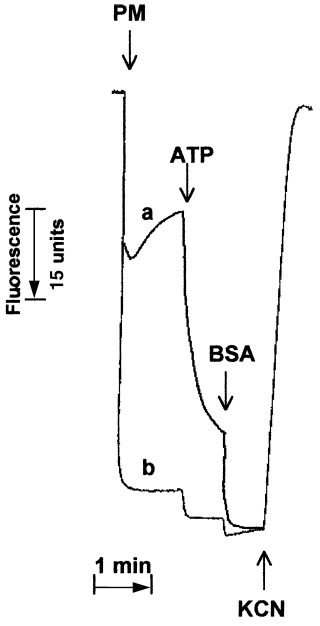

Fig. 1. Membrane potential of mitochondria isolated from potato tubers stored for 7 days at 4°C (line a) or 28°C (line b). Potato mitochondria (PM, 0.5 mg/ml) were added to the standard medium in the presence of 5 μM safranin. ATP (2 mM), 0.1% BSA and 1 mM KCN were added where indicated.

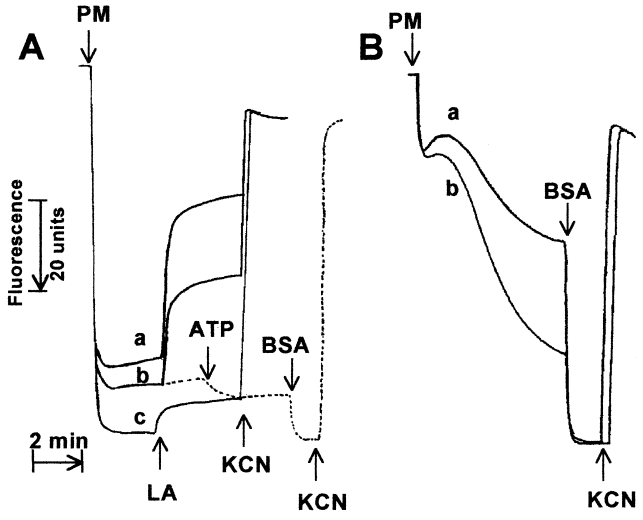

Fig. 2. LA-induced uncoupling of potato tuber mitochondria. Lines a and b of panel A represent the $\Delta\Psi$ of mitochondria (0.5 mg/ml) isolated from potatoes stored for 7 days at 4°C and 28°C, respectively, and line c represents an experiment with mitochondria isolated from fresh potatoes. All mitochondria were incubated in standard medium containing oligomycin (1.2 μg/mg of protein), propranolol (300 μM) and atractyloside (7.0 μM). LA (10 μM), 0.1% BSA and 1 mM KCN were added where indicated. The dashed line represents an experiment in which 2 mM ATP and 0.1% BSA were added in the absence of exogenous LA. In panel B, mitochondria (0.5 mg/ml) isolated in the presence of BSA were added to the same medium, but containing 10 μM LA. KCN (1 mM) and 0.1% BSA were added where indicated. Lines a and b represent experiments with mitochondria from potatoes stored at 4°C and 28°C, respectively.

and BSA on the $\Delta\Psi$ of these mitochondria. To minimize the involvement of the F_0F_1-ATP synthase, ADP/ATP carrier, and plant inner membrane anion channel [16] in the results, the experiments were performed in incubation medium containing specific inhibitors of these carriers (oligomycin, atractyloside and propranolol, respectively). Fig. 2A shows that under these experimental conditions mitochondria from fresh potatoes (line c) developed a $\Delta\Psi$ larger than that observed for potatoes stored at 28°C (line b) or 4°C (line a). The addition of 10 μM LA to these mitochondria caused an immediate decrease in $\Delta\Psi$ which increased from line c to line a, indicating that the LA uncoupling effect increased with aging and was stimulated by low temperature. The dashed line shows an experiment in which ATP and BSA were added sequentially to mitochondria from potatoes stored at 28°C. As with the results in Fig. 1, the coupling effects of ATP and BSA were also seen when potato mitochondria were incubated in reaction medium containing oligomycin, atractyloside and propranolol. It should be mentioned that although atractyloside completely blocks the ATP/ADP exchange, it inhibits the ATP/ADP antiporter-mediated uncoupling by FA by no more than 40% [19]. The experiment in Fig. 2B shows that under these conditions, potato mitochondria isolated in the presence of BSA (to deplete contaminant FA) displayed different sensitivities to exogenous 10 μM LA added to the medium prior to the mitochondria. Thus in the presence of LA, the membrane potential was much smaller in mitochondria isolated from potatoes stored at 4°C (line a). The addition of BSA brought $\Delta\Psi$ to similar levels in both preparations. These results support the interpretation that the LA uncoupling effect is, in-

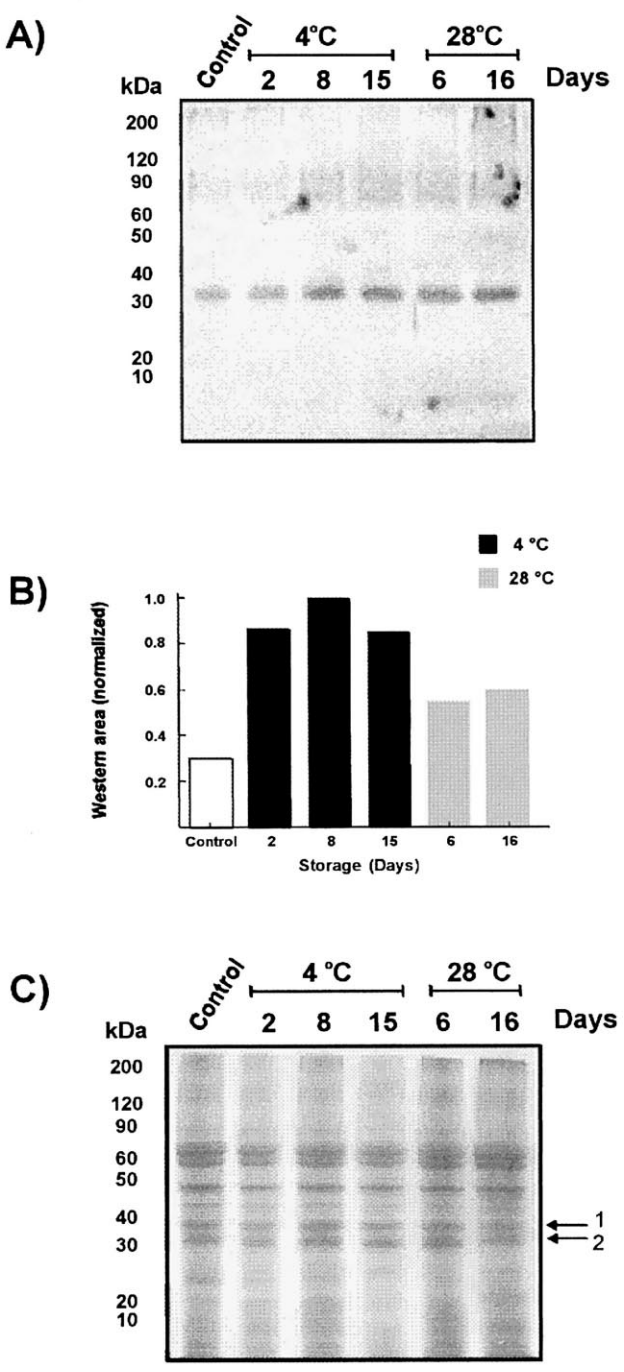

Fig. 3. Immunodetection of PUMP in potato tubers stored at 4°C or 28°C using anti-PUMP polyclonal antibodies. A: Immunoblot analysis of total mitochondrial proteins from potato tubers stored at 4°C or 28°C at various times. Values (kDa) at the left are for the prestained protein markers. B: Corresponding densitometric analysis of the immunoblot shown in A. The column labeled 'Control' represents an experiment with fresh potatoes. The densitometric analysis of the immunoblot was normalized to the densitometric results of the corresponding SDS-PAGE (C) using bands 1 and 2 as indicated.

deed, mediated by PUMP and that PUMP activity, and possibly content, increases with aging and low temperature. To evaluate this possibility, we separated the proteins from these mitochondria by SDS-PAGE (Fig. 3C) and performed immunoblot analysis using antibodies raised against potato PUMP. As expected, the amount of PUMP, visualized as a ~32 kDa band (Fig. 3A), increased as a function of storage time and was stimulated by low temperature as revealed by the immunoblot densitometry shown in Fig. 3B. After 2 days of storage at 4°C, the PUMP content increased almost three times and peaked after 8 days. When the potatoes were stored at 28°C, the PUMP content increased linearly during the 16 days of analysis. These results imply that the expression of PUMP is induced by aging and is stimulated by low temperature, and that, as with its protein counterpart in BATM, potato PUMP may play a role in plant thermogenesis. It should be considered that although these polyclonal antibodies were raised against a highly purified 32 kDa PUMP, the presence of a low quantity of contaminating ATP/ADP antiporter cannot be totally excluded. In this regard, we must recall that the ATP/ADP carrier, which may also mediate FA uncoupling [5] and be induced by cold acclimation in animals [20–22], could be contributing to the increase in the protein bands observed in Fig. 3 and in the mitochondrial uncoupling observed in Figs. 1 and 2.

To our knowledge, this is the first evidence that the level of PUMP increases in potatoes after exposure to cold. This finding agrees with those showing that a low temperature induces the expression of the StUCP and AtPUMP genes that encode uncoupling proteins in potato [14] and *A. thaliana* [15], respectively. In addition, the ability of PUMP to decrease the generation of reactive oxygen by potato mitochondria [23] suggests an important physiological role for this protein in preserving genome integrity and in conserving the post-harvesting intactness of reserve material in potato tubers.

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