Journal of Bioenergetics and Biomembranes, Vol. 35, No. 3, June 2003 (© 2003)

Stimulation of Potato Tuber Respiration by Cold Stress is Associated With an Increased Capacity of Both Plant Uncoupling Mitochondrial Protein (PUMP) and Alternative Oxidase

Fagoni Fayer Calegario,[1] Ricardo Guanaes Cosso,[1] Marcia Marilia Fagian,[1] Fernanda Vasconcelos Almeida,[2] Wilson Figueiredo Jardim,[2] Petr Ježek,[3] Paulo Arruda,[4] and Anibal Eugênio Vercesi[1,5]

Received February 11, 2003; accepted March 13, 2003

The CO_2 evolution of intact potato tubers (*Solanum tuberosum*, L., var. "Bintje") was analyzed during a 10-day period of their warm ($25 \pm 2^{\circ}C$) or cold ($5 \pm 1^{\circ}C$) storage, to evaluate cold-stress effects on expression and activities of plant uncoupling mitochondrial protein (PUMP) and alternative oxidase (AOX). CO_2 evolution rates were analyzed at 20°C, to reflect their possible capacities. The 20°C CO_2 production declined from 13 to 8 mg kg^{-1} h^{-1} after 2 days of warm storage and then (after 3 to 7 days) decreased from 8 to 6.5 mg kg^{-1} h^{-1}. In contrast, 20°C CO_2 evolution did not change after the first day of cold storage, increased up to 14.5 mg kg^{-1} h^{-1} after 2 days, and decreased to about 12 mg kg^{-1} h^{-1} after 3 to 7 days of cold storage. Cold storage increased PUMP expression as detected by Western blots and led to elevated capacities of both PUMP (44%) and CN-resistant AOX (10 times), but not the cytochrome pathway. Since we found that cold storage led to about the same mitochondrial respiration of 40 nmol O_2 min^{-1} mg^{-1} attributable to each of the respective proteins, we conclude that both AOX and PUMP equally contribute to adaptation of potato tubers to cold.

KEY WORDS: Whole potato tuber respiration; potato tuber mitochondria; plant uncoupling mitochondrial protein; alternative oxidase; cold stress.

INTRODUCTION

Energy dissipation in plant mitochondria can be mediated by two processes, via a redox potential dissipating system, the alternative oxidase (AOX), that branches from the main respiratory chain at the level of ubiquinone (Affourtit *et al.*, 2001a; Vanlerberghe and McIntosh, 1997); and by a proton electrochemical potential dissipating system, the plant uncoupling mitochondrial protein (PUMP) (Ježek *et al.*, 2001; Vercesi, 2001; Vercesi *et al.*, 1995). Both systems lead to the same final effect, i.e., to a decrease in the yield of ATP synthesis, which is inevitably linked to an increase in heat production. It

Key to abbreviations: AOX, alternative oxidase; BHAM, benzohydroxamate; BSA, bovine serum albumine; CN, cyanide; DTT, dithiothreitol; EGTA, ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; LA, linoleic acid; PUMP, plant uncoupling mitochondrial protein; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; Tris, tris [hydroxymethyl] amino-methane; UCP, uncoupling protein.

[1]Departamento de Patologia Clínica, NMCE, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-970, Campinas, SP, Brazil.

[2]Laboratório de Química Ambiental, Instituto de Química, Universidade Estadual de Campinas, Campinas, SP, Brazil.

[3]Department of Membrane Transport Biophysics, No. 75, Institute of Physiology, Academy of Sciences, Vídeňská 1083, Prague, Czech Republic.

[4]Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, 13083-970, Campinas, SP, Brazil.

[5]To whom correspondence should be addressed; e-mail: anibal@obelix.unicamp.br.

0145-479X/03/0600-0211/0

has been proposed that the activity of these proteins, in addition of being involved in metabolic regulation (Sluse *et al.*, 1998a), may also result in a decrease in reactive oxygen species (ROS) production (Kowaltowski *et al.*, 1998; Möller, 2001; Popov *et al.*, 1997; Wagner and Moore, 1997).

AOX catalyzes quinol-oxygen oxidation/reduction that is not linked to proton pumping and consequently does not generate a proton electrochemical gradient. The enzyme is not sensitive to cytochrome pathway inhibitors such as cyanide, antimycin A, or myxothiazol, but is inhibited by primary hydroxamic acids such as benzohydroxamate (BHAM), and in some tissues it can be stimulated by α-keto acids such as pyruvate (Day *et al.*, 1994; Millar *et al.*, 1993). In addition, AOX activity can be regulated by the reduction state of the enzyme, the oxidized dimer being less active than the reduced form (Umbach and Siedow, 1993; Umbach *et al.*, 1994). On the other hand, Vercesi *et al.* (1995) have demonstrated for the first time the existence of an uncoupling protein (PUMP) in potato tuber mitochondria. Subsequently, the biochemical and physiological properties of PUMP from different plant tissues have been characterized (Almeida *et al.*, 1999; Costa *et al.*, 1999; Jarmuszkiewicz *et al.*, 1998, 2000; Ježek *et al.*, 1996, 1997, 2000; Kowaltowski *et al.*, 1998; Nantes *et al.*, 1999; Sluse *et al.*, 1998a,b). The genes encoding PUMP1 (Ito, 1999; Laloi *et al.*, 1997; Maia *et al.*, 1998) or PUMP2 isoform (Watanabe *et al.*, 1999) have been also described. Recently, we have expressed the PUMP1 gene of *Arabidopsis thaliana* in *Escherichia coli*, isolated this recombinant protein, and characterized its phenotype after its reconstitution into liposomes (Borecký *et al.*, 2001). These functional and genetic approaches together provided a strong support that this protein is a true uncoupling protein and represents a plant counterpart of animal UCP2 or UCP3.

Despite the thermodynamic necessity of AOX and PUMP function resulting always in heat generation, the thermogenic role of AOX has been characterized only in specialized plant tissues, such as spadix of *Araceae* (Affourtit *et al.*, 2001a). For PUMP, a thermogenic role awaits investigations. In general, a thermogenic role can be manifested only at high-speed energy dissipation. However, plants are usually considered not to respire fast enough to generate heat (Breindenbach *et al.*, 1997). Nevertheless, a slow heat release must be concomitant to other relevant PUMP and AOX functions such as metabolic regulation (Affourtit *et al.*, 2001a; Ježek *et al.*, 2001; Sluse *et al.*, 1998a; Vanlerberghe and Mclntosh, 1997) and prevention of excessive ROS formation (Kowaltowski *et al.*, 1998; Möller, 2001; Popov *et al.*, 1997; Wagner and Moore, 1997). Among mammalian UCPs, the thermogenic role of UCP1 has been demonstrated only in brown adipose tissue of newborn, cold-acclimated, and hibernating mammals (Nicholls, 1979), but thermogenic roles of UCP2 (hypothetically in fever) or UCP3 have not yet been established (Ježek, 2002; Ricquier and Bouillaud, 2000). A thermogenic role of PUMP has been suggested to provide a logical adaptation to cold. However, no direct evidence was provided. The speculations come from observations of cold-stress-stimulated transcription of PUMP genes in potato root, flowers, and plant (*St*UcP gene, Laloi *et al.*, 1997) and in *Arabidopsis thaliana* (*At*PUMP1 gene, Maia *et al.*, 1998). Cold stress is also well known to activate AOX in potatoes (Zhou and Solomos, 1998) and other plants (Mclntosh *et al.*, 1998; Vanlerberghe and Mclntosh, 1997).

Since relatively little information is available on the PUMP function in intact tissues, we have analyzed, whether the increased expression of PUMP resulting from cold-storage (Nantes *et al.*, 1999) is paralleled by the increased respiration rate of the intact tubers. Our experiments hence attempted to indicate uncoupling in intact potato tubers and its possible increase when a plausible transcriptional upregulation of PUMP is induced by cold. Indeed, we have found that cold stress stimulates the respiration of intact potato tubers as well as the capacity of both PUMP and AOX but not of the cytochrome pathway. Since cold storage led to about the same mitochondrial respiration of 40 nmol O_2 min^{-1} mg^{-1} attributable to each of the respective protein, we conclude that both AOX and PUMP may equally contribute to adaptation of potato tubers to cold.

MATERIALS AND METHODS

Vegetable Material

Potato tubers (*Solanum tuberosum*, L. var. "Bintje") were obtained from a commercial source 3 days after harvest (referred here as fresh potatoes). Between the harvest and the use they were stored at 25°C. Tubers without visible defects and similar volume were selected. One group was dark-stored at 5 ± 1°C and another one at room temperature (25 ± 2°C). Groups of 16 potatoes were removed from storage to perform the measurement of intact potato tuber respiration and another group of 1.2 kg was removed from storage to extract mitochondria.

Isolation of Potato Tuber Mitochondria and Potential ($\Delta\Psi$) Monitoring

Mitochondria were isolated by conventional differential centrifugation, as previously described (Beavis and

Vercesi, 1992). Mitochondrial electrical transmembrane potential ($\Delta\Psi$) was estimated as a function of safranine O fluorescence decrease (Moore and Bonner, 1982), recorded on a model F-4500 Hitachi spectrofluorometer (Hitachi Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 5 nm. 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 mM $MgCl_2$, 2.5 mM potassium phosphate, 5 mM potassium-succinate, 2 μg mL^{-1} oligomycin, 300 μM propranolol (to inhibit the plant inner membrane anion channel (PIMAC), Beavis and Vercesi, 1992), 7 μM atractyloside, 10 μM linoleic acid (LA) and 0.1 mM ATP. Additions of 5 mM ATP, 0.1% BSA, and 1 mM potassium cyanide (KCN) were done. The results are representatives of at least three experiments.

Measurement of Respiration of Isolated Mitochondria

Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH). PUMP capacity was measured using 1.3 mL of a standard incubation medium (28°C) containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer, pH 7.2, 10 μM linoleic acid, 0.33 mM EGTA, 1 mM $MgCl_2$, 2.5 mM potassium phosphate, 5 mM potassium succinate (plus 5 μM rotenone), 2.5 μg oligomycin (mg protein)$^{-1}$, 300 μM propranolol, 2 μM atractyloside, 2 mM BHAM with 0.5 mg of mitochondrial protein. BSA 0.5% plus 5 mM ATP were added to inhibit PUMP activity. To ensure complete activation of succinate dehydrogenase, we added 0.1 mM ATP. The results presented are mean values of at least two experiments using the mitochondrial preparations isolated from the potato tubers stored during 4 days at 5 ± 1 or 25 ± 2°C.

The capacity of AOX was measured using the incubation medium described above without BHAM, plus 1 mM dithiothreitol (DTT) and 0.15 mM pyruvate to activate the alternative pathway and 0.5% BSA to inhibit PUMP activity. Additions of 2 mM KCN served for inhibition of the cytochrome (Cyt) pathway and 2 mM BHAM for inhibition of AOX.

In order to compare O_2 consumption (total respiration, CN-resistant respiration, and CN-sensitive respiration) by mitochondria isolated from potatoes stored at two temperatures (5 ± 1 or 25 ± 2°C) during 1–10 days, experiments were made in a completely randomized factorial design (storage temperature $\times$ storage time) and simple analysis of variance (ANOVA) was performed. As the interaction effect between storage temperature and storage time was significant, the Tukey's method was used to compare O_2 consumption of potato tubers according to the storage temperature at each time of storage and the time of storage for each storage temperature during the experiment. Significant differences were established at 1 or 5% level of significance ($p < 0.01$ or 0.05, respectively).

Measurement of Respiration of Intact Potato Tubers

Four groups (four repetitions) of four potato tubers were used in each treatment (storage temperature and storage time). Therefore, 16 tubers were used for the evaluation of the respiration rate after harvest and during the postharvest storage under refrigeration or at room temperature. A group of four potato tubers was hermetically enclosed inside a 3.4-L respiration vessel attached to a CO_2 detector. The atmosphere inside the respiration vessel was homogenized by an electric fan and pulled by a peristaltic pump, and the CO_2 released by the respiration of potato tubers was detected conductometrically (Calegario *et al.*, 2001). By calculating the difference between final and initial CO_2 concentration it was possible to determine CO_2 production per kilogram of potato tubers per hour.

Experiments were made in a completely randomized factorial design (storage temperature $\times$ storage time) and ANOVA was performed with the General Linear Model procedure of the Statistical Analysis System (SAS Institute, 1990). Duncan's method was used to compare the respiration rate of potato tubers according to the storage temperature during the experiment. Tukey's method was used to compare the respiration rate of potatoes stored under refrigeration and potatoes stored at room temperature. Significant differences were established at 5% level of significance ($p < 0.05$).

SDS-PAGE and Immunoblotting of PUMP

Up to 60 μg of mitochondrial protein per lane (concentration determined according to Greenberg and Craddock, 1982) was solubilized in the sample buffer (5% [w/v] SDS, 60 mM Tris-HCl, pH 6.8, 10% glycerol, 0.004% [w/v] bromophenol blue, and 0.5% [v/v] β-mercaptoethanol) and boiled for 5 min. After a SDS-PAGE (12% resolving gel), the gel was soaked for 5 min in 10 mM Tris-base containing 100 mM glycine and 10% methanol. The proteins were electrotransferred to nylon membranes (Hybond N, Amersham) in a semidry blotting apparatus (Pharmacia) and the membranes were blocked overnight at 5°C in TBS (20 mM Tris, pH 7.4, 137 mM

NaCl) plus 0.1% Tween 20, and 10% nonfat dry milk. The antibodies developed against the PUMP of *Arabidopsis thaliana* (Borecký *et al.*, 2001) were diluted (1:1000) in TBS and incubated for 12 h at 5°C. The blocked membranes were washed three times in the same buffer without milk, followed by incubation with an antirabbit IgG alkaline phosphatase conjugate 1:5000 for 1 h. Finally, the membranes were incubated for 5 min in the dark in a developing mixture containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and a solution CSPD 1:1000, and the bands were detected by chemiluminescence.

Measurements of Internal Temperature of Potato Tubers

In order to monitor the internal temperature of potato tubers during cold storage, a group of fresh potatoes was dark-stored at 5 ± 1°C, for 10 days. A thermometer (Taylor, Bi-Therm®) was inserted into two potatoes per day. After 10 min of stabilization, the temperatures of both potatoes were read and the average taken. After this procedure, both potatoes were discarded.

Chemicals

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

RESULTS

Standard 20°C CO_2 Production by Intact Potatoes Stored at Different Temperatures

The aim of the first experiments was to analyze whether expression of PUMP promoted by cold temperature is translated into the increased respiratory activity of the whole intact potato tubers. This was assessed by measurements of CO_2 evolution by the whole tuber at standard 20°C. Note that the aim was not to study temperature dependence of tuber respiration, but just to compare respiration of tubers with presumably two different levels of PUMP protein. Prior treatment of tubers involved either control treatment, identical to a warm storage at

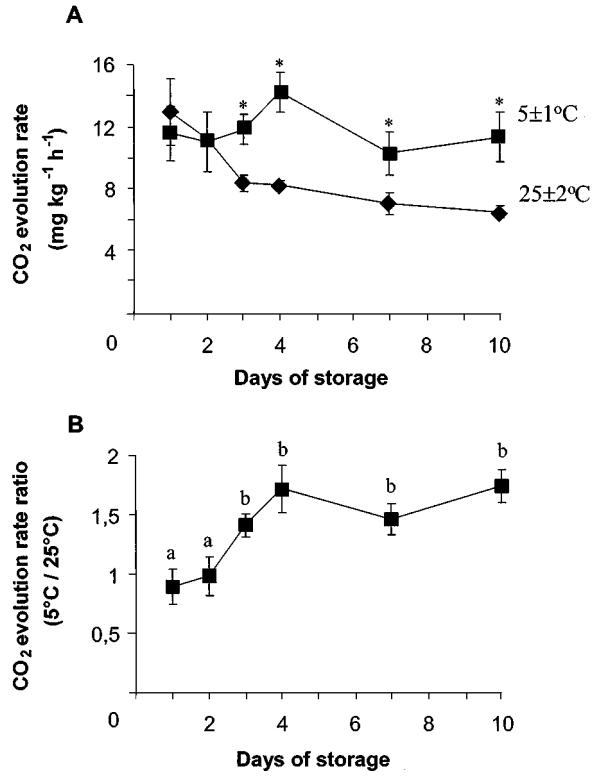

Fig. 1. (**A**) Respiration rate, measured at 20°C, of intact potato tubers during ten days of storage at 5 ± 1°C (■) or 25 ± 2°C (◆). Values of respiration rate are expressed in milligrams per kilogram per hour evolved CO_2. Each point represents the mean value and SD of four determinations, each one using a group of four intact potato tubers. Significant differences ($p < 0.05$) between cold- and warm-treatments are indicated by ∗. (**B**) Ratio between CO_2 evolution rates of intact potato tubers stored at 5 ± 1°C and those stored at 25 ± 2°C. Different letters indicate statistically different ($p < 0.01$) mean values.

25 ± 2°C, or cold-stress treatment, representing a cold storage at 5 ± 1°C during a period of up to 10 days. Figure 1(A) shows that the rate of 20°C CO_2 production sharply declined from 13 to 8 mg CO_2 kg^{-1} h^{-1} after 3 days of warm (control) storage at 25 ± 2°C. The control rates then decreased more slowly, from 8 to 6.5 mg CO_2 kg^{-1} h^{-1} after warm storage lasting 4 to 7 days. On the contrary, 20°C CO_2 evolution from cold-stressed potato tubers did not significantly change after a cold storage at 5 ± 1°C lasting for 1 to 3 days. Moreover, the 4-day cold storage led to an increased 20°C CO_2 evolution up to 14.5 mg CO_2 kg^{-1} h^{-1}. The 20°C CO_2 evolution rates were slightly declining from this maximum to about 12 mg CO_2 kg^{-1} h^{-1} after the cold storage lasting between 5 and 10 days.

The ratio between 20°C respiratory rates of potato tubers stored at low and room temperature increased from 0.9 up to 1.5–1.9 during the first 4 days of storage and

within an experimental error maintained this level after storage lasting for 5 to 10 days (Fig. 1(B)). We can conclude that cold stress applied to tubers most probably induced increased expression of PUMP or led to other adaptations manifested by the increased respiration after the recovery from the stress (after the transfer to 20°C).

The tubers stored at 5°C were monitored and we observed that the temperature inside the potatoes was equal to the temperature of the cold room and constant during all the period of storage. This shows that no heat evolution if existed could be detected most probably because of a high thermostatic capacity of the cold room.

PUMP Expression Upon Cold Stress of Potato Tubers

The expression of PUMP was estimated by Western blot analysis of mitochondria isolated from warm- or cold-treated potato tubers, as indicated by Fig. 2. Its upper panel shows the presence of PUMP antigen visualized using anti-*At*PUMP-antibodies. PUMP did not vary in the case of control (warm) storage at 25 ± 2°C lasting for 1 to 10 days. The cold storage, however, led to progressively increasing amount of PUMP antigen (lower panel of Fig. 2). Already after the 3 days of cold storage, PUMP expression was higher than in control potato tubers kept at room temperature. The maximum levels of PUMP antigen were found after 10 days of cold storage (Fig. 2).

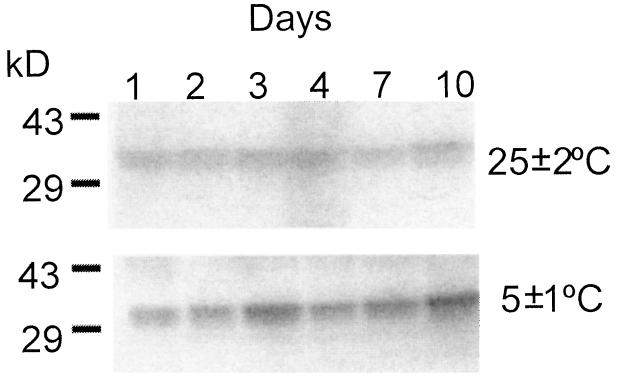

Fig. 2. Immunodetection of PUMP among total mitochondrial proteins from potato tubers stored at 5 ± 1 or 25 ± 2°C during 1–10 days. Proteins were electrophoresed, transferred to nylon membranes, and reacted with polyclonal antibodies against *Arabidopsis* PUMP1, as described in Materials and Methods section. The molecular mass markers appear on the left. All the steps for both temperatures were made simultaneously.

PUMP Functional Capacity in Mitochondria From Cold-Stressed Potato Tubers

In order to ascertain whether PUMP could be involved in the increased respiratory rate of the whole intact potato tubers subjected to cold stress, we analyzed the functional capacity of PUMP in mitochondria isolated from the tubers after the cold storage at 5 ± 1°C and from control tubers stored at 25 ± 2°C. The PUMP functional capacity has been taken as the sum of extent of BSA and ATP responses—the sum of their recoupling effects on mitochondria which were preincubated with 10 μM linoleic acid. Hence, the sum of the BSA-induced and ATP-induced increase in membrane potential ($\Delta\Psi$) of isolated mitochondria was followed. Note that also ATP at a low (0.1 mM) concentration was present from the beginning to activate succinate dehydrogenase from its cytoplasmic side (Affourtit *et al.*, 2001b) and atractylate was present to inhibit the FA-induced uncoupling mediated by the ADP/ATP carrier.

The cold storage lasting 4 days (Fig. 3(A)) yielded mitochondria responding well by recoupling because of the ATP addition as well as due to the subsequent addition of BSA. On the contrary, ATP had no recoupling effect on control mitochondria from warm-stored potato tubers. In these mitochondria the BSA-induced recoupling was much lower than after the cold stress. Even more difference between control and cold-stressed tuber mitochondria were accounted for 10-day cold storage (Fig. 3(A)). Whereas control mitochondria exhibited almost similar response to 4-day warm storage, the cold stress lasting for 10 days led to significantly increased extent of BSA-induced recoupling (Fig. 3(B)). ATP recoupled by a slight less extent than after the 4-day cold storage. One can consider these results as reflecting the higher amount of PUMP in mitochondria of cold-stressed potatoes.

Respiration monitoring under the conditions similar to those of Fig. 3(A) also indicated the possible PUMP increase upon the cold stress (Fig. 4). Additionally, the AOX inhibitor BHAM was present, in order to exclude the AOX contribution. The uncoupling induced by 10 μM LA, reflecting a possible PUMP activity, was monitored at state-4 respiration supported by succinate (plus rotenone, oligomycin, and BHAM). The extent of PUMP stimulation was related to the state with depleted fatty acids (depleted by BSA) and with ATP present (slope 2 minus slope 3 in Fig. 4). It is justified by the fact that after the LA addition (slope 2), the H^+ leak is negligible, since it was observed that LA-induced $\Delta\Psi$ decrease equals to the $\Delta\Psi$ drop due to phosphorylation of ADP (Almeida *et al.*, 1999). Mitochondria from 4-day cold-stressed potatoes exhibited LA-uncoupled rate of 114.5 nmol O_2 min^{-1}

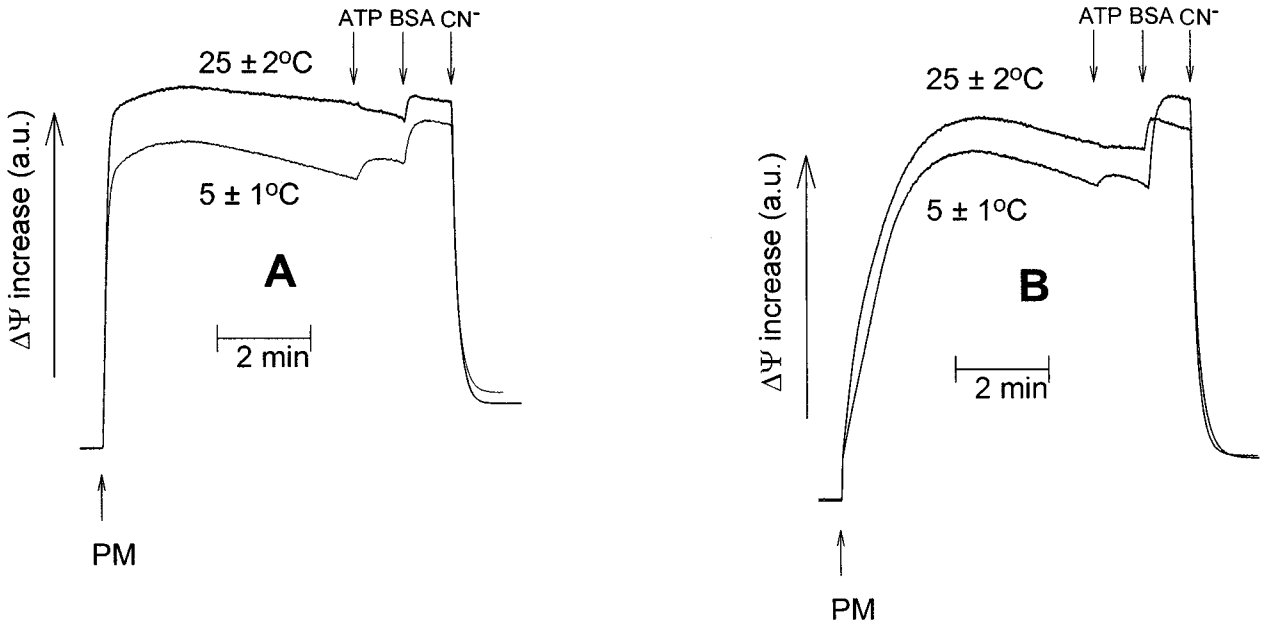

Fig. 3. Electrical transmembrane potential ($\Delta\Psi$) of mitochondria isolated from potato tubers stored for four (panel A) and 10 days (panel B) at 25 ± 2 or 5 ± 1°C. Potato mitochondria (0.5 mg mL^{-1}) were incubated in a standard medium (28°C) containing 2 μg oligomycin (mL protein)$^{-1}$, 7 μM atractyloside, 300 μM propranolol, 0.1 mM ATP, and 10 μM LA, as described in Materials and Methods section. Additions of 5 mM ATP, 0.1% BSA and 1 mM KCN were done where indicated. $\Delta\Psi$ was estimated as a function of changes in safranin O fluorescence (arbitrary units, here referred to as a.u.).

(mg protein)$^{-1}$, which is by 18% higher than in controls showing 97.1 nmol O_2 min^{-1} (mg protein)$^{-1}$. Respiratory rate sustained only by an H^+ leak (0.5% BSA plus 5 mM ATP, slope 3) was the same within the experimental error (66.2 and 63.3 nmol O_2 min^{-1} (mg protein)$^{-1}$ for cold stress and controls, respectively). Hence the resulting PUMP-sustained O_2 consumption (slope 2 minus slope 3 in Fig. 4) is 48.3 and 33.5 nmol O_2 min^{-1} (mg protein)$^{-1}$ for mitochondria from cold-stressed and control potato tubers, respectively. In conclusion, it reflects a 44% increase in PUMP capacity induced by the cold stress, a result that is in agreement with the degree of PUMP expression (Fig. 2) and PUMP-induced decrease in $\Delta\Psi$ (Fig. 3).

Note also, that the maximum uncoupled rates in the presence of FCCP were similar for cold stress and controls (155 and 153 nmol min^{-1} (mg^{-1} protein)$^{-1}$, respectively). This indicates that the cytochrome pathway is not affected by the cold stress.

Capacities of AOX in Isolated Mitochondria of Cold-Stressed Potato Tubers

We also measured the capacities of AOX pathway in mitochondria isolated from cold-stressed potato tubers and the warm-stored controls. The typical experiment is illustrated in Fig. 5, while the complete summary of the data is presented in Fig. 6. This assay reflects the AOX activity monitored as the CN-resistant respiratory rate at 2 mM KCN in state 4 (succinate plus rotenone and oligomycin) in the presence of the AOX activators pyruvate and DTT (Fig. 5). CN-resistant respiration was corrected for the residual low rates (1–2 nmol min^{-1} (mg protein)$^{-1}$) of O_2 consumption after the addition of 2 mM BHAM. The obtained pattern for the time course of cold storage and control warm storage is shown in Fig. 6. Figure 6, panel A, shows the total respiratory rate, the panel B indicates the CN-resistant respiration, hence the AOX capacity, whereas the panel C indicates the CN-sensitive respiration. Total respiratory rates were higher upon the cold stress and maintained levels above 80 nmol O_2 min^{-1} (mg protein)$^{-1}$, with exception of the 3-day cold storage (Fig. 6(A)). These results roughly match those obtained with the whole intact tubers (Fig. 1(A)), since the higher O_2 consumption was reached after 4 days of cold storage.

The AOX capacity was about 10 times higher for cold storage lasting from four to ten days, when compared to the warm-stored controls (Fig. 6(B)). It reached magnitudes about 40 nmol O_2 min^{-1} (mg protein)$^{-1}$. These results are in agreement with report of Zhou and Solomos (1998), who found that low temperatures sharply increased the AOX capacity in potato tuber mitochondria.

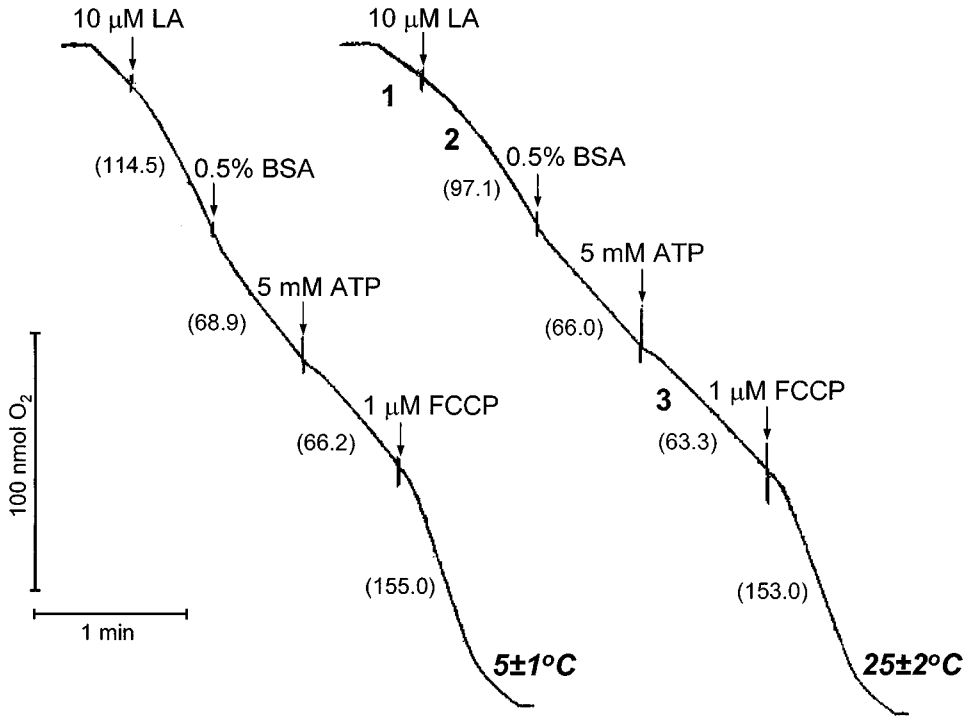

Fig. 4. Determination of PUMP-sustained respiration under standard conditions: Mitochondria protein (0.5 mg mL^{-1}) were incubated in a standard reaction medium (28°C) in the presence of 2.5 μg oligomycin (mg protein)$^{-1}$, 300 μM propranolol, 2 μM atractyloside, 0.1 mM ATP and 2 mM BHAM, as described in Materials and Methods sections. Additions of 10 μM linoleic acid (LA), 0.5% BSA, 5 mM ATP, and 1 μM FCCP were done where indicated. The numbers in parenthesis refer to the O_2-consumption rates in nmol min^{-1} (mg protein)$^{-1}$, as mean values of two determinations. Slope designation (in bold) refers to: **1**, state-4 respiration; **2**, state-4 respiration with 10 μM LA; **3**, coupled respiration with 0.5% BSA and 5 mM ATP.

The CN-sensitive respiration for mitochondria of cold-stressed potatoes was similar to controls kept at room temperature, except of 1-day storage (Fig. 6(C)). This is in agreement with the observed similar rates of FCCP-stimulated respiration (Fig. (4)) and again confirms that the cytochrome pathway is not responsible for the changes in respiratory rate that occurred after the cold stress.

DISCUSSION

It is well known that during cold acclimation periods, new proteins form to help the plant adapt to the altered environment (Guy, 1990; Kannerworff and van der Plas, 1994; Kolesnichenko *et al.*, 2000a,b). In this regard, we have previously observed that expression of PUMP was stimulated in potato tubers stored at 4°C when compared to those kept at 28°C (Nantes *et al.*, 1999). In this work we have extended these observations to monitoring of respiration of whole intact potato tubers, when simultaneously assaying for capacities of PUMP and AOX. By immunodetection we have confirmed an increasing amount of expressed PUMP upon the cold storage lasting up to 10 days. Similar data were previously obtained for AOX (Zhou and Solomos, 1998), who also observed a similar respiration increase when potato tubers were transferred from 10 to 1°C. They found that the rate of CO_2 output initially declined and was followed by a rapid increase reaching a peak of respiration rate approximately threefold higher than that observed at 10°C within 12 days. Kannerworff and van der Plas (1994) also observed higher O_2 consumption in tulip bulbs stored at 5°C than in the bulbs stored at 20°C.

In our measurements, the potato tuber respiration at 20°C has increased 1.5 to 1.9-fold after the cold storage lasting for at least 4 days at 5°C. In parallel, a high increase in PUMP antigen (Fig. 2) as well as a 44% increase in PUMP functional capacity occurred. AOX, which is known to have also the elevated expression under these conditions (Zhou and Solomos, 1998), increased its functional capacity about 10 times. Nevertheless, since we found that cold storage led to about the same

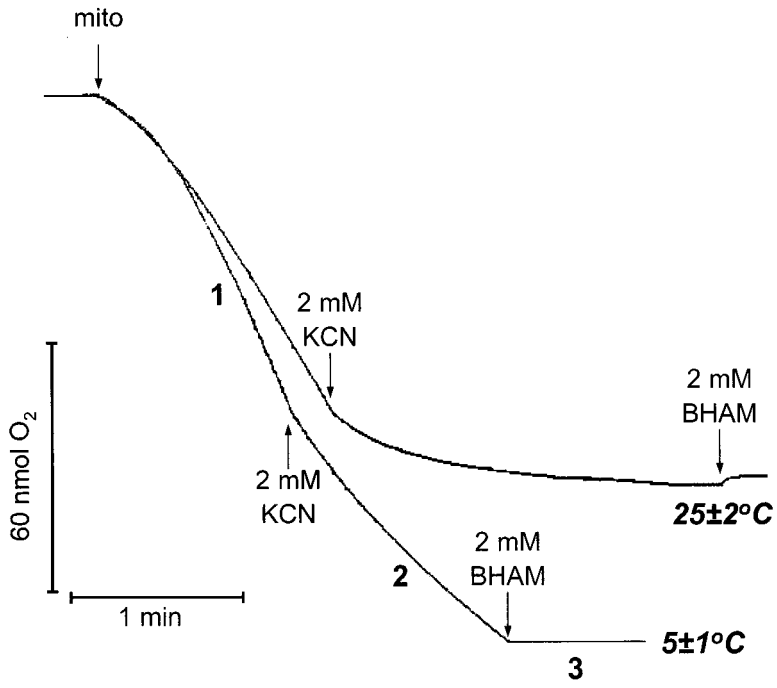

Fig. 5. Determination of AOX capacity in mitochondria isolated from potato tubers stored for 4 days at 5 ± 1 or 25 ± 2°C. Mitochondria (0.5 mg mL^{-1}) were incubated in a standard reaction medium (28°C) in the presence of 2.5 μg oligomycin (mg protein)$^{-1}$, 300 μM propranolol, 2 μM atractyloside, 0.1 mM ATP, 0.5% BSA, 1 mM DTT, and 0.15 mM pyruvate, as described in Materials and Methods section. Additions of 2 mM KCN and 2 mM BHAM were done where indicated. Slope **1** refers to the total respiration rate, a difference between slope **1** and slope **2** refers to CN-sensitive respiration and between slope **2** and slope **3** refers to AOX capacity.

mitochondrial respiration of 40 nmol O_2 min^{-1} mg^{-1} attributable to each of the respective proteins, we conclude that both AOX and PUMP may equally contribute to adaptation of potato tubers to cold. Their increase in capacity upon cold stress contrasts to the unchanged function of the cytochrome pathway upon cold stress (Figs. 4 and 6).

However, what we measured were the capacities of the respective proteins at standard conditions, but not their contributions to the increased respiration of intact potato tubers. We demonstrated quite well a correlation between the whole tuber respiration and capacities of PUMP and AOX, since they were reaching saturation after the 4 days of cold storage. Our conclusion that both AOX and PUMP may contribute equally was derived from their tested standard capacities. In vivo, however, various situations may occur which will modulate contribution of each of the two proteins. For example, for PUMP activation, aging will be more relevant (Nantes *et al.*, 1999), due to the aging-induced cleavage of polyunsaturated fatty acids (PUFAs), which on the contrary inhibit AOX (Sluse, 1998a). During aging PUFAs are likely to be cleaved by phospholipase c and subsequent cyclooxygenase reaction produces hydroperoxides and ROS, notably also PUFA radicals (Kumar and Knowles, 1993, 1996). In this regard, unpublished results from our group (M. Brandalise, I. G. Maia, J. Borecký, A. E. Vercesi and P. Arruda) demonstrate that overexpression of PUMP in transgenic tobacco plants led to a significant increase in tolerance to oxidative stress promoted by exogenous hydrogen peroxide as compared to nontransgenic control plants. Hence, we might consider that the purpose of the cold-stress-induced PUMP expression and activity is to balance the potentially increasing ROS production.

Our potato tubers were evaluated shortly after harvest. They were not yet stabilized in the postharvest period, since we observed a decrease in the rate of CO_2 evolution in the potato tubers stored at 25 ± 2°C. This may reflect a decrease in metabolic activity, which follows this postharvest period. If one assumes that such stabilization of metabolic activity (possibly a decrease in a substrate release, etc.) proceeds by equal or slower rate also in tubers stored at 5°C, it emphasizes even more the observed up to 1.9-fold increase in whole tuber respiration upon the cold stress. The tuber respiration is elevated, even if

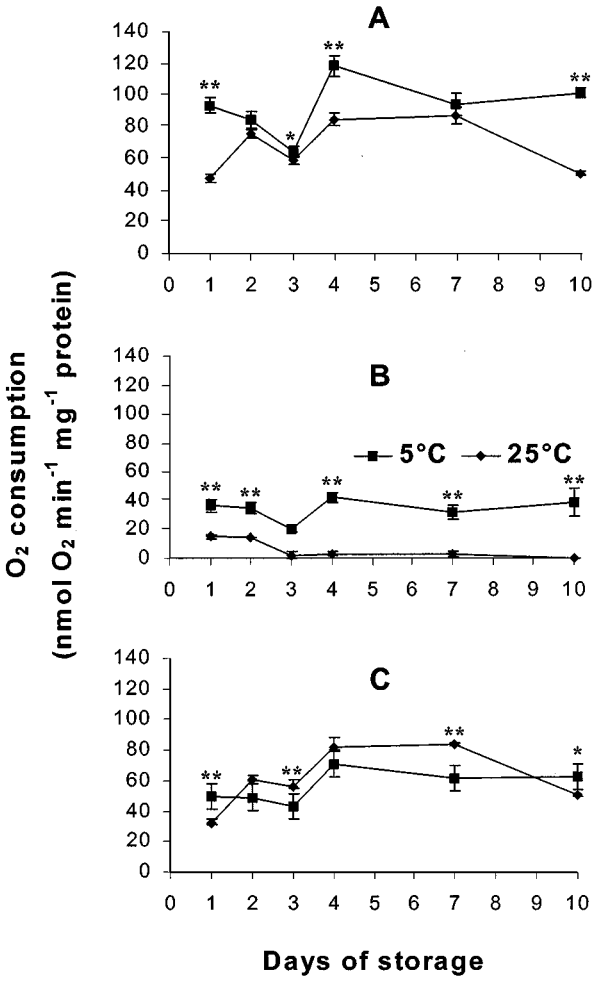

Fig. 6. O_2 consumption by potato mitochondria isolated from tubers stored for 1, 2, 3, 4, 7, and 10 days at $5 \pm 1°C$ (■) or $25 \pm 2°C$ (◆). The rates of O_2 consumption were measured in experiments such as those depicted in Fig. 5. (**A**) Total respiration; (**B**) CN-resistant respiration; (**C**) CN-sensitive respiration. Each point represents the average O_2 consumption (nmol O_2 min^{-1} (mg protein)$^{-1}$) of three experiments. Vertical lines indicate standard deviation of the measurements. Significant differences are indicated by ** when $p < 0.01$ and * when $p < 0.05$.

one can assume that substrate availability is limited. Related to this, it is interesting to note that PUMP activity in tomato fruits remained constant when the activity of mitochondrial ATP-ase was limited by the lower substrate availability (Sluse *et al.*, 1998b).

We may also discuss possible thermogenic roles of both AOX and PUMP. Since we clearly demonstrated their elevated capacities upon the cold stress, we suppose that the increase in whole tuber respiration had to be at least partly due to nonphosphorylating respiration. It would be against the thermodynamic law, if such a respiration did not produce heat. However, in the high thermostatic capacity of the cold room we were not able to detect a temperature increase during the whole period of storage. It also reflects the fact that any existing nonphosphorylating respiration had to be slow and unable to elevate temperature of bulk tissue (potato tubers) under practical storage conditions. In this regard, Moynihan *et al.* (1995) and Breindenbach *et al.* (1997) suggested that although most plants, with the notable exception of thermogenic plants, clearly do not produce enough metabolic heat to raise the temperature of bulk tissue. However, respiratory heat may have a pronounced local effect at the subcellular level and a localized increase in temperature around the individual mitochondrion may be of physiological importance (Moynihan *et al.*, 1995).

ACKNOWLEDGMENTS

This research was supported by grants from the following Brazilian agencies: Fundaçaõ de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Programa de Apoio a Núcleos de Excelência (FINEP/PRONEX), and Coordenação de A perfeiçoamento de Pessoal de Nível Superior (CAPES, PhD grant to F.F.C.). The authors thank Helymar Machado and Cleide Silva for providing the statistical analysis, Ivan Maia and Jiri Borecký for their helpful suggestions. PJ is supported by GACR grant No. 521/02/0183.

REFERENCES

Affourtit, C., Krab, K., and Moore, A. L. (2001a). *Biochim. Biophys. Acta* **1504**, 58–69.

Affourtit, C., Krab, K., Leach, G. R., Whitehouse, D. G., and Moore, A. L. (2001b). *J. Biol. Chem.* **276**, 32567–32574.

Almeida, A. M., Jarmuszkiewicz, W., Khomsi, H., Arruda, P., Vercesi, A. E., and Sluse, F. E. (1999). *Plant Physiol.* **119**, 1323–1329.

Beavis, A. D., and Vercesi, A. E. (1992). *J. Biol. Chem.* **267**, 3079–3087.

Borecký, J., Maia, I. V., Costa, A. D. T., Ježek P., Chaimovich, H., Andrade, P. B. M., Vercesi, A. E., and Arruda, P. (2001). *FEBS Lett.* **505**, 240–244.

Breidenbach, R. W., Saxton, M. J., Hansen, L. D., and Criddle, R. S. (1997). *Plant. Physiol.* **114**, 1137–1140.

Calegario, F. F., Cosso, R. G., Almeida, F. V., Vercesi, A. E., and Jardim, W. F. (2001). *Postharvest Biol. Technol.* **22**, 249–256.

Costa, A. D. T., Nantes, I. L., Ježek, P., Leite, A., Arruda, P., and Vercesi, A. E., (1999). *J. Bioenerg. Biomembr.* **31**, 527–533.

Day, D. A., Millar, A. H., Wiskich, J. T., and Whelan, J. (1994). *Plant Physiol.* **106**, 1421–1427.

Greenberg, C. S., and Craddock, P. R. (1982). *Clin. Chem.* **28**, 1725–1726.

Guy, C. L. (1990). *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* **41**, 178–223.

Ito, K. (1999). *Plant Sci.* **149**, 167–173.

Jarmuszkiewicz, W., Almeida, A. M., Sluse-Goffart, C. M., Sluse, F. E., and Vercesi, A. E. (1998). *J. Biol. Chem.* **273**, 34882–34886.

Jarmuszkiewicz, W., Almeida, A. M., Vercesi, A. E., Sluse, F. E., and Sluse-Goffart, C. (2000). *J. Biol. Chem.* **275**, 13315–13320.

Ježek, P., Costa, A. D. T., and Vercesi, A. E. (1996). *J. Biol. Chem.* **271**, 32743–32748.
Ježek, P., Costa, A. D. T., and Vercesi, A. E. (1997). *J. Biol. Chem.* **272**, 24272–24278.
Ježek, P., Borecký, J., Žáčková, M., Costa, A. D. T., and Arruda, P. (2001). *Biosci. Rep.* **21**, 237–245.
Ježek, P., Žáčková, M., Košařová, J., Rodrigues, E. T. S., Madeira, V. M. C., and Vicente, J. A. F. (2000). *J. Bioenerg. Biomembr.* **32**, 549–561.
Kannerworff, W. A., and van der Plas, L. H. W. (1994). *Plant Sci.* **104**, 31–38.
Kolesnichenko, A. V., Zykova, V. V., and Voinikov, V. K. (2000a). *J. Therm. Biol.* **25**, 203–209.
Kolesnichenko, A. V., Zykova, V. V., Grabelnych, O. I., Sumina, O. N., Pobezhimova, T. P., and Voinikov, V. K. (2000b). *J. Therm. Biol.* **25**, 245–249.
Kowaltowski, A. J., Costa, A. D. T., and Vercesi, A. E. (1998). *FEBS Lett.* **425**, 213–216.
Kumar, G. N. M., and Knowles, N. R. (1993). *Plant Physiol.* **102**, 115–124.
Kumar, G. N. M., and Knowles, N. R. (1996). *Physiol. Plant* **97**, 228–236.
Laloi, M., Klein, M., Riesmeier, J. W., Muller-Róber, B., Fleury, C., Boillaud, F., and Ricquier, D. (1997). *Nature* **389**, 135–136.
Maia, I. G., Benedetti, C. E., Leite, A., Turcinelli, S. R., Vercesi, A. E., and Arruda, P. (1998). *FEBS Lett.* **429**, 403–406.
McIntosh, L., Eichler, T., Gray, G., Maxwell, D., Nickels, R., and Wang, Y. (1998). *Biochim. Biophys. Acta* **1365**, 278–284.
Millar, A. H., Wiskich, J. T., Whelan, J., and Day, D. A. (1993). *FEBS Lett.* **329**, 259–262.
Möller, I. M. (2001). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 561–591.
Moore, A. L., and Bonner, W. D. (1982). Measurements of membrane-potentials in plant-mitochondria with the safranine method. *Plant Physiol.* **70**, 1271–1276.
Moynihan, M. R., Ordentlich, A., and Raskin, I. (1995). *Plant Physiol.* **108**, 995–999.
Nantes, I. L., Fagian, M. M., Catisti, R., Arruda, P., Maia, I. G., and Vercesi, A. E. (1999). *FEBS Lett.* **457**, 103–106.
Nicholls, D. G. (1979). *Biochim. Biophys. Acta* **549**, 1–29.
Popov, V. N., Simonian, R. A., Skulachev, V. P., and Starkov, A. A. (1997). *FEBS Lett.* **415**, 87–90.
Ricquier D., and Bouillaud F. (2000). *Biochem. J.* **345**, 161–179.
SAS Institute (1990). *SAS User's Guide: Basics*, version 5. SAS Institute, Cary, NC.
Sluse, F. E., Almeida, A. M., Jarmuszkiewicz, W., and Vercesi, A. E. (1998a). *FEBS Lett.* **433**, 237–240.
Sluse, F. E., Jarmuszkiewicz, W., Almeida, A. M., Vercesi, A. E., Hryniewiecka, L., and Sluse-Goffart, C. (1998b). In *Plant Mitochondria: From Gene to Function* (Moller, I. M., Gardeström, P., Glimelius, K., and Glaser, E., eds.), Backhuys Publishers, Leiden, The Netherlands, pp. 519–523.
Umbach, A. L., and Siedow, J. N. (1993). *Plant Physiol.* **103**, 845–854.
Umbach, A. L., Wiskich, J. T., and Siedow, J. N. (1994). *FEBS Lett.* **348**, 181–184.
Vanlerberghe, G. C., and McIntosh, L. (1997). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 703–734.
Vercesi, A. E. (2001). *Biosci. Rep.* **21**, 195–200.
Vercesi, A. E., Martins, I. S., Silva, M. A. P., Leite, H. M. F., Cuccovia, I. M., and Chaimovich, H. (1995). *Nature* **375**, 24.
Wagner, A. M., and Moore, A. L. (1997). *Biosci. Rep.* **17**, 319–33.
Watanabe, A., Nakazono, M., Tsutsumi, N., and Hirai, A. (1999). *Plant Cell Physiol.* **40**, 1160–1166.
Zhou, D. B., and Solomos, T. (1998). *Physiol. Plant* **104**, 255–265.