Reactive Oxygen Species Production by Potato Tuber Mitochondria Is Modulated by Mitochondrially Bound Hexokinase Activity

Juliana Camacho-Pereira², Laudiene Evangelista Meyer², Lilia Bender Machado, Marcus Fernandes Oliveira, and Antonio Galina*

Instituto de Bioquímica Médica, Programa de Biofísica e Bioquímica Celular and Programa de Biologia Molecular e Biotecnologia, Universidade Federal do Rio de Janeiro, Laboratório de Bioenergética e Fisiologia Mitoccondrial, Cidade Universitária, Rio de Janeiro 21941–590, Brazil

Potato tuber (Solanum tuberosum) mitochondria (PTM) have a mitochondrially bound hexokinase (HK) activity that exhibits a pronounced sensitivity to ADP inhibition. Here we investigated the role of mitochondrial HK activity in PTM reactive oxygen species generation. Mitochondrial HK has a 10-fold higher affinity for glucose (Glc) than for fructose ($K_{m,GC} = 140 \mu M$ versus $K_{m,FR} = 1,375 \mu M$). Activation of PTM respiration by succinate led to an increase in hydrogen peroxide ($H_2O_2$) release that was abrogated by mitochondrial HK activation. Mitochondrial HK activity caused a decrease in the mitochondrial membrane potential and an increase in oxygen consumption by PTM. Inhibition of Glc phosphorylation by mannoheptulose or GlcNAc abrogated by mitochondrial HK activation. Mitochondrial HK activity caused a decrease in the mitochondrial membrane potential formed across the inner mitochondrial membrane. High $\Delta \psi_m$ was shown to stimulate ROS production when the ETS is predominantly in a reduced state (i.e. when NADH, FADH$_2$, and O$_2$ are present in abundance but ADP or Pi levels are low). This condition is reached in resting metabolic states after a full oxidation of Glc or fatty acids. Stimulating electron flow by decreasing $\Delta \psi_m$ either by the use of uncouplers or by coupling respiration to ATP synthesis, slows the ROS generation rate (Boveris and Chance, 1973; Korshunov et al., 1997). It has been

1 This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro.
2 These authors contributed equally to the article.
* Corresponding author; e-mail galina@bioqmed.ufrj.br.

The author responsible for the distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Antonio Galina (galina@bioqmed.ufrj.br).

www.plantphysiol.org/cgi/doi/10.1104/pp.108.129247

Production of reactive oxygen species (ROS) is an unavoidable consequence of aerobic respiration (Chance et al., 1979). The mitochondrial electron transport system (ETS) is the major site of ROS production in mammalian and nonphotosynthesizing plant cells (Puntarulo et al., 1991; Halliwell and Gutteridge, 2007). Depending on the mitochondrial respiratory states, a small portion of the consumable oxygen is partially reduced to generate ROS (Skulachev, 1996; Liu, 1997; Turrens, 1997; Möller, 2001; Considine et al., 2003; Smith et al., 2004). In plants, the mono-electronic reduction of oxygen by ETS leads to the production of superoxide radicals (O$_2^{-}\cdot$) that can be dismutated by SOD, producing hydrogen peroxide ($H_2O_2$), and further decomposed by catalase and/or ascorbate-glutathione peroxidase cycles (Mölle, 2001). An imbalance between the ROS production and antioxidant defenses can lead to an oxidative stress condition. Increased levels of ROS may be a consequence of the action of plant hormones, environmental stress, pathogens, or high levels of sugars and fatty acids (Bolwell et al., 2002; Couée et al., 2006; Gechev et al., 2006; Liu et al., 2007; Rhoads and Subbaiah, 2007). These conditions may lead to storage deterioration or impairment of seedling growth decreasing on crop yield. To avoid the harmful accumulation of ROS or to fine tune the steady-state levels of ROS, various enzymatic systems control the rate of ROS production in mitochondria (Schreck and Baeuerle, 1991; Möller, 2001).

Mitochondrial ROS production is highly dependent on the membrane potential ($\Delta \psi_m$) generated by the proton gradient formed across the inner mitochondrial membrane. High $\Delta \psi_m$ was shown to stimulate ROS production when the ETS is predominantly in a reduced state (i.e. when NADH, FADH$_2$, and O$_2$ are present in abundance but ADP or Pi levels are low). This condition is reached in resting metabolic states after a full oxidation of Glc or fatty acids. Stimulating electron flow by decreasing $\Delta \psi_m$ either by the use of uncouplers or by coupling respiration to ATP synthesis, slows the ROS generation rate (Boveris and Chance, 1973; Korshunov et al., 1997). It has been
observed that in isolated potato tuber (*Solanum tuberosum*) mitochondria (PTM) the uncoupling protein (referred to as PUMP in plants, or UCP in animals) causes a small decrease in $\Delta \Psi_m$ when this proton carrier protein is activated by the presence of anionic fatty acids, a condition that blocks ROS generation (Vercesi et al., 1995, 2006). Nucleotides, such as ATP, antagonize this effect (Considine et al., 2003; Vercesi et al., 2006). On the other hand, fluctuations in free hexose levels due to environmental or developmental conditions (Morrell and ap Rees, 1986; Geigenberger and Stitt, 1993; Renz and Stitt, 1993) lead to variations in the oxygen consumption rate in heterotrophic tissues of plant (Brouquiise et al., 1991; Dieuaide et al., 1992). As a result, ROS-producing pathways may be either stimulated or repressed (Couée et al., 2006). Unlike PUMP activity, which is activated by an excess of free fatty acids, a specific mechanism for mitochondrial ROS production caused by an excess of hexose remains elusive.

The metabolism of free hexoses begins by their phosphorylation in a reaction catalyzed by the hexokinase (HK):

$$\text{hexose} + \text{ATP} \rightarrow \text{hexose-6-P} + \text{ADP}$$

HK is a ubiquitous enzyme found in many organisms. In plants, the binding mechanism of HK to the outer mitochondrial membrane is not fully established, but some reports indicate that it may differ considerably from those properties described for mammal cells (Dry et al., 1983; Miernyk and Dennis, 1983; Rezende et al., 2006). It has been shown that in several mature and developing plant tissues, multiple HK isoforms are expressed with different kinetic properties and subcellular localizations. The HKs are found in cytosol, bound to the mitochondrial membrane, or in stroma of plastids in plant cells (Miernyk and Dennis, 1983; Galina et al., 1995; Damari-Weissler et al., 2007). Beyond its obvious role in glycolysis regulation, HK activity may also function as a sugar sensor, triggering a signal transduction pathway in plants (Rolland et al., 2006).

In mammals, HK types I and II are associated with the mitochondrial outer membrane through the voltage-dependent anion channel (VDAC) and adenine nucleotide transporter (ANT). These associations were found in tissues with a high energy demand, such as heart, brain, and tumor cells (Arora and Pedersen, 1988; BeltrandelRio and Wilson, 1992; Wilson, 2003). In addition, recent evidence in mammalian cells has shown that binding of HK to VDAC located at the outer mitochondrial membrane is somehow involved in the protection against proapoptotic stimuli (Nakashima et al., 1986; Gottlob et al., 2001; Vander Heiden et al., 2001; Pastorino et al., 2002; Cesar and Wilson, 2004). Similar observations were reported for tobacco (*Nicotiana tabacum*) plant mitochondrial HK (mt-HK; Kim et al., 2006). However, it has been shown that drugs such as the fumigicide clotrimazole and the anesthetic thiopental, which promptly disrupt the association between mt-HK and VDAC in mammalian mitochondria, are unable to promote this effect in maize (*Zea mays*) root mitochondria (Rezende et al., 2006). These observations suggest a different type of association of mt-HK with plant mitochondria. The binding of mt-HK with mitochondria in many plants involves a common N-terminal hydrophobic membrane anchor domain of about 24 amino acids that is related to the membrane targeting, but the exact mechanism of association is unknown (Damari-Weissler et al., 2007).

Recently, our group demonstrated that mt-HK activity plays a key preventive antioxidant role by reducing mitochondrial ROS generation through a steady-state ADP recycling mechanism in rat brain neurons. The mitochondrial ADP recycling leads to a decrease in $\Delta \Psi_m$ coupled to the synthesis of ATP by oxidative phosphorylation (da-Silva et al., 2004; Meyer et al., 2006).

Although plant HK is known to fulfill a catalytic function, the role of mt-HK activity in the regulation of both mitochondrial respiration and ROS production is unknown. Recently, an authentic HK activity was detected in PTM (Graham et al., 2007) and its involvement in potato tuber glycolysis suggested, but its involvement in PTM ROS generation was not explored. We then raise the hypothesis that HK bound to PTM would contribute to produce a steady-state ADP recycling that regulates ROS formation. However, whether this association is capable of controlling the rate of ROS generation in plant mitochondria is unknown. Here, we aim to investigate the role of mt-HK activity in PTM physiology. The data indicate that mt-HK activity plays a key role as a regulator of ROS levels in respiring plant tissues exposed to high hexose levels.

**RESULTS**

**An Authentic HK Activity Is Associated with PTM**

Previous studies have shown that HK is bound to mitochondria in mammalian tissues, in different plant species (Galina et al., 1995; da-Silva et al., 2001; Wilson, 2003; Meyer et al., 2006; Claeyssen and Rivoal, 2007), and even in PTM (Graham et al., 2007). To check whether a particulate HK activity from potato tuber is also associated with this organelle, in Table I we determined the activities of HK, oligomycin-sensitive $F_0F_1$-ATPase, a mitochondrial enzyme, and Glc-6-P dehydrogenase (G6PDH), a cytosolic and plastid enzyme (Dennis and Green, 1975; Miernyk and Dennis, 1983) in different fractions of potato tuber homogenates. Clearly, the majority of HK activity is concentrated in the particulate fraction (P12) of potato tuber (Table I). As the $F_0F_1$-ATPase and the G6PDH activities were also substantial in P12 fraction, a further separation step was carried out on the P12 fraction by using a self-generated Percoll gradient method, producing...
two main fractions. One was enriched in plastids (fraction 1) and the other was enriched in mitochondria (fraction 2; Neuburger et al., 1982). Accordingly, the F$_0$F$_1$ATPase activity was enriched 13-fold in fraction 2 of the Percoll gradient as compared to the homogenate (Table I). The HK activity was present in both plastid and mitochondrial fractions from the Percoll gradient, being enriched 8-fold in this fraction and only 2-fold in the plastid fraction (Table I). The low G6PDH activity detected in the mitochondrial fraction indicates a very low degree of cross contamination between these fractions. Almost all particulate G6PDH activity was associated with the plastid fraction (Table I). These data show that an authentic HK activity is associated with PTM.

**Kinetic Properties of Potato Tuber mt-HK and Dependence of Activity on Oxidative Phosphorylation**

The next step was to evaluate the activity of mt-HK after addition of ATP and Glc using intact, Percoll-purified PTM (Fig. 1). The mt-HK activity was detected immediately after addition of 1 mM ATP and 5 mM Glc (Fig. 1A, traces 1 and 2), but was progressively inhibited by more than 90% after 2 min. However, the mt-HK activity could be restored by the inclusion of

---

**Table 1. Mitochondrial location of potato tuber particulate HK activity**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Enzyme Activities</th>
<th>Total Protein in Fraction</th>
<th>Total HK Activity per Fraction$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F$_0$F$_1$ATPase</td>
<td>HK</td>
<td>N.D.</td>
</tr>
<tr>
<td>Homogenate</td>
<td>6.4 ± 2.2 (n = 7)</td>
<td>9.7 ± 2.4 (n = 8)</td>
<td>16.4 ± 1.9 (n = 4)</td>
</tr>
<tr>
<td>S12$^c$</td>
<td>8.4 ± 2.5 (n = 7)</td>
<td>2.6 ± 0.6 (n = 8)</td>
<td>25.5 ± 1.2 (n = 6)</td>
</tr>
<tr>
<td>P12$^d$</td>
<td>22.4 ± 8.1 (n = 6)</td>
<td>32.1 ± 3.5 (n = 8)</td>
<td>30.8 ± 3.8 (n = 6)</td>
</tr>
<tr>
<td>P12 after Percoll gradient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 1</td>
<td>5.7 ± 0.5 (n = 4)</td>
<td>20.2 ± 7.7 (n = 7)</td>
<td>32.2 ± 2.9 (n = 4)</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>83.9 ± 22.6 (n = 7)</td>
<td>75.8 ± 16 (n = 8)</td>
<td>0.3 ± 0.1 (n = 6)</td>
</tr>
</tbody>
</table>

$^a$The F$_0$F$_1$ATPase, HK, and G6PDH activities were measured as described in "Materials and Methods." The reaction time was 15 min and the temperature was 28°C. The activities represent mean ± se of, at least three independent preparations, as shown in parentheses. $^b$Total HK activity (100%) was considered to be the sum of the HK activities recovered in S12 plus P12 fractions. $^c$S12 is the supernatant of 12,000 g/10 min. $^d$P12 is the particulate pellet (12,000g/10 min).

---

**Figure 1.** Mt-HK activity is dependent on respiration, kinetic properties of ADP inhibition of mt-HK activity, and hexose phosphorylation. A, The time course of G6P production by mt-HK in PTM after addition of 5 mM Glc and 1 mM ATP is shown in traces 1, 2, and 3. Isolated PTM, 0.2 mg/mL, was added to respiration buffer containing 1 unit/mL G6PDH and 1 mM β-NAD$^+$.

---

Copyright © 2009 American Society of Plant Biologists. All rights reserved.
succinate, an oxidizable substrate that supports the conversion of ADP to ATP during oxidative phosphorylation, indicating that inhibition observed for mt-HK was due to the ADP accumulated by the mt-HK reaction (Fig. 1A, traces 1 and 2). The mt-HK sensitivity to ADP inhibition was confirmed when 0.3 mM ADP was added to the reaction medium prior to starting the mt-HK reaction with ATP plus Glc (Fig. 1A, trace 3). The mt-HK reaction rate in this case was much lower than that observed in the first 2 min in the absence of ADP (Fig. 1A, trace 1). The addition of succinate reestablished the maximal rate of mt-HK activity (Fig. 1A, trace 3). The authenticity of mt-HK activity was confirmed when its specific competitive inhibitor, 50 mM mannoheptulose (MHP), was added to the reaction medium (Fig. 1A, trace 2). No oxidative phosphorylation-supported mt-HK activity was observed in the presence of oligomycin (a specific inhibitor of F0F1ATPase), atractyloside (a specific inhibitor of adenine-nucleotide translocator ANT), or in the absence of succinate (data not shown). In addition, these oxidative phosphorylation inhibitors had no effect on PTM mt-HK activity (data not shown).

Figure 1B shows the ADP dependence for mt-HK inhibition using Glc or Fru as substrate. ADP caused a half inhibition of mt-HK activity in the micromolar range with Glc (IC50 = approximately 40–70 μM), but much more ADP was needed to cause a similar degree of inhibition (IC50 = approximately 400–700 μM) with Fru as substrate. This result suggests that the affinity of mt-HK for Glc and Fru is different and the inhibitory activity of ADP depends on the substrate used. In fact, the affinity of mt-HK from PTM was higher for Glc (K_M 0.14 mM) than for Fru (K_M 1.4 mM). The mt-HK activity measured with saturating concentrations of hexoses was practically the same (Fig. 1C).

Figure 2. The rate of oxygen consumption and membrane potential (ΔΨ_m) are modulated by mt-HK activity. A. Oxygen uptake was measured using the respiration buffer described in “Materials and Methods.” Numbers indicate the rate of oxygen consumption (nmol O_2 min⁻¹ mg ptn⁻¹). Mito, 0.2 mg/mL Percoll-purified PTM protein was added to the respiration medium. B. The ΔΨ_m was measured with fluorescence quenching of safranine O. The numbers indicated are the final ADP concentration, in micromolar, added to the cuvette in each pulse. After the inclusion of 200 μM ADP, a pulse of 5 mM Glc was added, followed by 50 mM MHP to inhibit mt-HK activity. When indicated, 100 μM atractyloside (Atr) was added to the reaction medium. Suc (in A and B), 10 mM succinate. In C is shown a higher magnification of the ΔΨ_m trace in B. The figure shows a representative experiment. The membrane potential and the oxygen consumption measurements were repeated with at least 15 independent PTM preparations.

Potato Tuber mt-HK Activity Modulates Oxygen Consumption, ΔΨ_m, and H₂O₂ Release

The experiments shown in Figure 1 indicated that PTM respiration is important to maintain the mt-HK activity free of the ADP inhibition. In the next set of experiments, we evaluated the effect of the mt-HK activity on several mitochondrial functional parameters (Figs. 2, 3, and 4). During PTM respiration, the addition of small amounts of ADP (0.15 mM) accelerated the rate of O₂ consumption (state 3; Fig. 2A). When all the ADP was converted to ATP, the respiration rate decreased, returning to the resting state (state 4), but a further addition of Glc permanently stimulated the O₂ consumption (Fig. 2A). Based on previous data from our group (da-Silva et al., 2004; Meyer et al., 2006), Glc-induced O₂ consumption in PTM is a result of ADP generated by the mt-HK reaction being transported by the ANT to the mitochondrial matrix and then being utilized as substrate by the FₐFₐ,ATP synthase, decreasing the ΔΨ_m and leading to an acceleration of the electron flux in the respiratory electron chain. This can be confirmed by ΔΨ_m measurements showing that sequential ADP additions transiently dissipated the ΔΨ_m (Fig. 2, B and C), reaching an estimated ATP concentration of 545 μM. Inclusion of Glc after all ADP pulses promoted a small, but consistent decrease in ΔΨ_m (about 3% of maximal value; Figs. 2, B and C, and 4A), which was reversed by the inclusion of 50 mM MHP (Fig. 2, B and C). An increase in Glc concentration to 10 mM further decreased the ΔΨ_m (Fig. 2C), and interruption of the ADP recycling mechanism by ANT using atractyloside promoted an increase in the ΔΨ_m (Fig. 2C). These data show that the mt-HK activity is able to modulate the proton motive force in PTM.
It has been established that mitochondrial H$_2$O$_2$ formation is strongly dependent on high $\Delta \Psi_m$ values (Korshunov et al., 1997). In Figure 3 we observed substantial H$_2$O$_2$ release after induction of respiration by succinate and ATP. The addition of 1 mM ADP strongly reduced H$_2$O$_2$ release, showing that a small decrease in $\Delta \Psi_m$ due to activation of state 3 respiration, is the main mechanism by which mitochondrial H$_2$O$_2$ release is prevented (Fig. 3, left trace). A similar blockage in H$_2$O$_2$ release was observed when ADP was replaced by 5 mM Glc (Fig. 3, right trace). This was reversed by either mt-HK inhibitors such as MHP (Fig. 3) or N-acetylglucosamine (NAG) (Fig. 4C) by oxidative phosphorylation poisons such as oligomycin or atracyloside, two inhibitors that promote an increase in $\Delta \Psi_m$ in the presence of oxidizable substrates (Figs. 2, and 4, A and B). These experiments demonstrate that mt-HK activity is able to modulate the $\Delta \Psi_m$ and H$_2$O$_2$ release in PTM by an ADP-recycling mechanism through ANT/F$_{0}$F$_{1}$ATP synthase activities. The regulation of H$_2$O$_2$ release from PTM by mt-HK is attained regardless if the source of ATP for the mt-HK is external (Fig. 4B) or from oxidative phosphorylation (Fig. 4A).

Glc Dependence for mt-HK Activity Is Inversely Related to the Rate of H$_2$O$_2$ Release by PTM

The mt-HK activity and the rate of H$_2$O$_2$ release by PTM were measured simultaneously with increasing amounts of Glc (Fig. 5A). We observed an inverse relation between mt-HK activity and mitochondrial H$_2$O$_2$ release. The IC$_{50}$ to inhibit H$_2$O$_2$ release was approximately 20 $\mu$M Glc (Fig. 5A). The dependence on mt-HK activity to abrogate the H$_2$O$_2$ release was confirmed in Figure 5B, when 25 mM NAG, a competitive inhibitor of mt-HK was included in the assay.
medium. Under this condition, the IC₅₀ for Glc to inhibit H₂O₂ release rose to about 800 µM. Importantly, a half-maximum inhibition of the mt-HK activity was able to maintain the rate of H₂O₂ release at about 15% of its maximal value (Fig. 5C).

Comparison of the Preventive Antioxidant Role of mt-HK and PUMP Activities in PTM

The rate of H₂O₂ release, oxygen consumption, and ΔΨₘ, levels were titrated with increasing amounts of a proton ionophore, carbonyl p-trifluoromethoxyphenylhydrazone (FCCP), or with the PUMP activator linolenic acid (LA; Vercesi et al., 2006; Fig. 6). In the titration with FCCP (Fig. 6A), it became apparent that a small decrease in the maximal ΔΨₘ (about 3% of arbitrary units of fluorescence of safranine O) by 700 nM FCCP (Fig. 6A, white circles) promoted a 2-fold stimulation of oxygen consumption (Fig. 6A, white stars) and a complete inhibition of H₂O₂ release (Fig. 6A, white triangles). The effect of maximal mt-HK activity compared with these parameters was determined and the set point gives a 3% decrease in ΔΨₘ, expressed as arbitrary units of fluorescence of safranine O (black circle), an increase of 1.8-fold in oxygen consumption (black star), and a reduction of at least 95% in H₂O₂ release (black triangle; see Fig. 6A, vertical, dotted line for set point).

In an attempt to compare the impact of mt-HK activity with that of PUMP activation on the bioenergetic parameters evaluated above, we next measured the effect of increasing amounts of LA, either with (Fig. 6B)
6B) or without fatty acid-free bovine serum albumin (faf-BSA; a protein that sequesters LA; Fig. 6C). The IC_{50} for LA to inhibit H_{2}O_{2} release was about 30 μM in the presence of faf-BSA (Fig. 6B, white triangles). Under this specific condition mt-HK activation exerted an even more powerful effect on H_{2}O_{2} release than did PUMP activation (Fig. 6C, black triangle). The IC_{50} for LA to inhibit H_{2}O_{2} release when faf-BSA was omitted from the reaction medium (Fig. 6C) was about 4 μM, confirming the ability of this fatty acid to activate PUMP. In this condition, 30 μM LA almost completely blocked H_{2}O_{2} release, similar to mt-HK activation (Fig. 6C, white and black triangles).

**Prevention of H_{2}O_{2} Release by PTM mt-HK Requires Specific Enzyme Association to Mitochondria**

To evaluate whether a specific location of mt-HK on mitochondria is required to prevent H_{2}O_{2} release we next measured the rate of H_{2}O_{2} release in PTM using two alternative hexose substrates, Glc or Fru at 0.5 mM (Fig. 7A). Mt-HK activity using Fru as substrate is much lower when compared with Glc, as expected from the apparent affinities of these two substrates (Fig. 1C). In fact, when 0.5 mM Fru was added to the reaction mixture, a negligible effect was observed on the rate of H_{2}O_{2} release, contrasting with the strong reduction in the rate of H_{2}O_{2} release when 0.5 mM Glc was used as substrate (Fig. 7A). Mt-HK activity using Fru as substrate is much lower when compared with Glc, as expected from the apparent affinities of these two substrates (Fig. 1C). In fact, when 0.5 mM Fru was added to the reaction mixture, a negligible effect was observed on the rate of H_{2}O_{2} release, contrasting with the strong reduction in the rate of H_{2}O_{2} release when 0.5 mM Glc was used as substrate (Fig. 7A). Mt-HK activity using Fru as substrate is much lower when compared with Glc, as expected from the apparent affinities of these two substrates (Fig. 1C). Regardless of the substrate used, oligomycin promptly increased the H_{2}O_{2} release by impairing the ADP recycling activity mediated by mt-HK.

In contrast to PTM mt-HK activity, soluble yeast (*Saccharomyces cerevisiae*) HK exhibits a high affinity for Fru with an apparent K_{m} of 0.7 mM (Avigad and Englard, 1968; Bernard, 1975). Taking advantage of this difference in kinetic properties between the two enzymes, we next compared the effect of soluble yeast HK activation, with that of activating the endogenous PTM mt-HK by using Fru as substrate and measuring the rate of H_{2}O_{2} release. The rationale was that at 0.5 mM Fru, PTM mt-HK would not be active, thus not producing ADP, but addition of soluble yeast HK, which has a higher affinity for Fru, would promptly catalyze this reaction. Thus, under this condition, the ADP produced from the HK reaction comes only from the exogenous HK activity. Clearly, increasing amounts of the soluble yeast-HK decreased the rate of H_{2}O_{2} release in PTM (Fig. 7A). On comparing at the levels of soluble yeast HK activity required to impair H_{2}O_{2} release in PTM with the activity of endogenous mt-HK, we observed a difference of at least two orders of magnitude (Fig. 7B). This result led us to conclude that specific location of mt-HK at the outer PTM membrane is absolutely essential to improve ADP delivery for the oxidative phosphorylation, decreasing PTM H_{2}O_{2} release.

**Glc Impairs H_{2}O_{2} Release in Potato Tuber Slices**

Potato tuber slices incubated with Glc showed a rate of oxygen consumption 1.6-fold higher than those without Glc or with Suc (Fig. 8A). The potato tuber slices treated with FCCP showed oxygen consumption 2.2-fold higher than that observed for the control (Fig. 8A). To ascertain whether these increased rates in O_{2} consumption were related to the rate of H_{2}O_{2} release, we measured the accumulation of H_{2}O_{2} in potato tuber slices medium. The response was the opposite to that observed for the rate of respiration by potato tuber slices in each condition (Fig. 8B and C). The rate H_{2}O_{2} release by potato tuber slices incubated with Glc showed a rate of oxygen consumption 1.6-fold higher than those without Glc or with Suc (Fig. 8A). The potato tuber slices treated with FCCP showed oxygen consumption 2.2-fold higher than that observed for the control (Fig. 8A). To ascertain whether these increased rates in O_{2} consumption were related to the rate of H_{2}O_{2} release, we measured the accumulation of H_{2}O_{2} in potato tuber slices medium. The response was the opposite to that observed for the rate of respiration by potato tuber slices in each condition (Fig. 8B and C). The rate H_{2}O_{2} release by potato tuber slices incubated with Glc showed a rate of oxygen consumption 1.6-fold higher than those without Glc or with Suc (Fig. 8A). The potato tuber slices treated with FCCP showed oxygen consumption 2.2-fold higher than that observed for the control (Fig. 8A). To ascertain whether these increased rates in O_{2} consumption were related to the rate of H_{2}O_{2} release, we measured the accumulation of H_{2}O_{2} in potato tuber slices medium. The response was the opposite to that observed for the rate of respiration by potato tuber slices in each condition (Fig. 8B and C).
release in disc slices was 3-fold lower when the slices were incubated with 5 mM Glc compared to the rate measured in the absence or in the presence of Suc, a nonphosphorylatable sugar for mt-HK (Fig. 8B, black circles and white triangles against white circles). When H$_2$O$_2$ release by potato tuber slices was measured in the presence of Glc and 50 mM NAG, an inhibitor of mt-HK, the rate of H$_2$O$_2$ release tended to be higher than that measured only in the presence of Glc (Fig. 8B, black triangles). This result is in accordance with those shown in Figures 3, 4, and 5 using isolated PTM. The H$_2$O$_2$ release measured in the presence of FCCP and without Glc was as low as that recorded for the potato slices incubated with 5 mM Glc without FCCP (Fig. 8B, white squares). These results indicate that the preventive effects of Glc on H$_2$O$_2$ release mediated by the mt-HK activity observed in isolated PTM are working in a similar manner in the potato tuber slices and are associated with increased O$_2$ consumption.

DISCUSSION

Changes in free hexose levels by carbohydrate starvation and reverse feeding lead to variations in the respiration rate in heterotrophic plant tissues (Brouquisse et al., 1991; Dieuaide et al., 1992). These free hexose fluctuations may correspond to certain environmental or developmental conditions where sugar availability may be limiting (Morrell and ap Rees, 1986; Geigenberger and Stitt, 1993; Renz and Stitt, 1993; Couée et al., 2006) and are associated with ROS generation.

High Glc levels are toxic in several models because of increased release of ROS by Glc autooxidation and metabolism (Couée et al., 2006). The leakage of ROS from mitochondria is as important in nonphotosynthesizing plant cells as it is in mammalian cells (Skulachev, 1996, 1997; Møller, 2001). In animal cells, high Glc concentrations can lead to activation of NADPH oxidase, which is one of the sites responsible for Glc toxicity (Bonnefont-Rousselot, 2002). However, it has been proposed that ROS release by mitochondria via the respiratory chain is a causal link between high Glc levels and the main pathways responsible for cell damage (Russell et al., 1999, 2002; Nishikawa et al., 2000). In this regard, our group recently demonstrated that mt-HK activity plays a central role in preventing mitochondrial ROS generation through a steady-state ADP recycling in rat brain (da-Silva et al., 2004). High Glc levels in plants may not trigger mitochondrial ROS formation as in mammals for the following reasons: (1) plant mt-HK activities are not strongly inhibited by hexose-6-P as they are in mammals (Renz and Stitt, 1993; Galina et al., 1995; da-Silva et al., 2001, 2004; Meyer et al., 2006); (2) plant mt-HK activities are strongly inhibited by ADP (Fig. 1B; Galina et al., 1995; da-Silva et al., 2001). These observations may explain the plant cell tolerance to mitochondrial ROS formation due to high sugar concentration availability compared to mammalian cells (Nishikawa et al., 2000; Brownlee, 2001; Couée et al., 2006). Thus, the ADP recycling mt-HK activity would play a key preventive antioxidant role by keeping lower ΔΨ$_m$ and ROS generation levels in mitochondria.

In this work we observed mt-HK activity (Table I) in isolated PTM, in agreement with a recent report (Graham et al., 2007). The mt-HK activity found in PTM is highly dependent on oxygen consumption, because the activation of mitochondrial respiration by succinate restores the activity levels of the enzyme (Fig. 1A). On the other hand, the activity of mt-HK is able to promote an acceleration of oxygen consumption after the conversion of ADP to ATP (Fig. 2A). This would ensure that the HK could respond rapidly to changes in the cellular demand for Glc-6-P (G6P), which is known to be a key intermediate in several metabolic pathways sensitive to the ADP to ATP ratio.
including glycolysis, Suc synthesis, pentose-P pathway, and cellulose biosynthesis.

As previously observed for maize mt-HK, mt-HK activity from PTM is also much more sensitive to ADP inhibition in the micromolar range when the substrate is Glc than with Fru (Fig. 1B; Galina et al., 1995, 1999; da-Silva et al., 2001). This result suggests that the affinity of mt-HK for Glc and Fru is different. In fact this was shown in Figure 1 and is in accordance with the kinetic behavior of mt-HK from maize seedling roots (Galina et al., 1999).

 Because atractyloside and oligomycin impair the decrease of ΔΨm and the preventive role in H2O2 formation sustained by the mt-HK reaction, we can conclude that this mt-HK activity is able to promote modulation of electron flux in ETS via a mechanism of ADP recycling through ANT: F0F1 ATP synthase complex (Figs. 2 and 4). This mechanism of control of H2O2 formation was previously observed in rat brain mitochondria that contain large amounts (more than 80% of total tissue content; Wilson, 2003; da-Silva et al., 2004) of mt-HK activity, but it had not been described so far for plant mitochondria.

Besides the classical antioxidant enzymes (superoxide dismutase, catalase, and ascorbate-glutathione peroxidase), two systems have been identified as participants in controlling pro- and antioxidant balance in plants, the alternative oxidase (AOX) and the uncoupling protein (PUMP). Ultimately, these two systems suggest that the access to ADP is substantially increased by mt-HK in PTM and in potato tuber slices. Several lines of evidence indicate that, in addition to its activity, the localization of mt-HK is relevant for mitochondrial respiration (Moore and Jöbsis, 1970; BeltrandelRio and Wilson, 1991; Galina et al., 1995). This possibility would be explained in mammalian mitochondria by the mt-HK binding site in the VDAC-ANT complex. According to our previous study, mt-HK localization seems to be critical for performing its preventive antioxidant activity, as the ADP would be rapidly delivered through the VDAC-ANT complex to the F0F1 ATP synthase, which phosphorylates it at the expense of ΔΨm (da-Silva et al., 2004). Although the binding mechanism of mt-HK to plant mitochondrial outer membranes is not yet fully established (Rezende et al., 2006), the data presented in Figure 7 is similar to data from previous study in rat brain mitochondria that demonstrated that the mt-HK activity is more effective in stimulating respiration in the presence of Glc than its nonbindable chymotrypsin-treated mt-HK form (Moore and Jöbsis, 1970; BeltrandelRio and Wilson, 1991).
These similarities between mt-HK and StUCP in the response with regard to ROS formation may indicate that potato tubers have complementary mechanisms against oxidative damage induced by respiration in heterotrophic plant tissues. In conditions of higher oxidative metabolism fueled by hexoses leading to increased ROS formation, the mt-HK activity would play a predominant role as a preventive mechanism. On the other hand, when the rate of fatty acid β-oxidation is increased, the UCP becomes the main mechanism to prevent the accumulation of ROS. In plant tissues, we cannot exclude the possible operation of AOX in detoxifying the formation of superoxide anions. However, in potato tuber under no abiotic stress, the AOX activity levels are low (Calegario et al., 2003; Considine et al., 2003). In our experimental conditions the cyanide-resistant respiration is less than 3% when using succinate or NADH as oxidizable substrates, and about 10% when using pyruvate plus malate as substrates (data not shown).

CONCLUSION

Thus, besides its involvement in general plant sugar metabolism, in Glc sensing (Rolland et al., 2006), and in the regulation of programmed cell death (Kim et al., 2006), we propose that mt-HK plays a specific role in generating ADP to support oxidative phosphorylation, thereby avoiding an ATP synthesis-related limitation of respiration and subsequent H$_2$O$_2$ release in plants.

MATERIALS AND METHODS

Chemicals and Biological Materials

ADP, ATP, FCCP, horseradish peroxidase, rotenone, safranine O, MHP, NAG, yeast (Saccharomyces cerevisiae) HK, faf-BSA, β-NAD$^+$, LA, oligomycin, and G6PDH from Leuconostoc mesenteroides were purchased from Sigma-Aldrich. Percoll was from Amersham Biosciences. Ampex Red was purchased from Invitrogen. All other reagents were analytical grade. Potato tubers (Solanum tuberosum) were purchased from a local supermarket.

Isolation of PTM by Self-Generated Percoll Gradient

PTM were obtained as previously described (Neuburger et al., 1982) using a cold extraction buffer containing: 10 mM HEPES/Tris pH 7.4, 0.3 mM mannitol, 2 mM EGTA, 5 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 20 mM β-mercaptoethanol, and 0.1 g % (w/v) faf-BSA. The homogenate was strained through eight layers of cheesecloth and centrifuged at 3,000 g at 4°C for 30 min. The supernatant was centrifuged at 12,000 g at 4°C for 10 min. The supernatant (S12) was used to determine enzymatic activities. The mitochondrial pellet (P12) was resuspended in 5 mL of ice-cold extraction buffer and layered in Hitachi P50 centrifuge tubes containing 35 mL of cold extraction buffer containing 28% (v/v) Percoll and centrifuged at 40,000 g at 4°C for 30 min. After centrifugation, three major bands were obtained in sequence; the first band (fraction 1) at the top and the middle band (fraction 2) were used for further PTM isolation or for enzymatic activity determinations. Mitochondrial activity markers were found to be enriched in fraction 2. The mitochondrial fraction was removed and diluted with extraction buffer without Percoll and centrifuged twice at 12,000 g at 4°C for 10 min. The final pellet was resuspended in 0.6 mL of extraction buffer and kept in an ice-water bath. The final protein concentration varied from 10 to 20 mg/mL.

Enzyme Assays and Kinetic Parameters

The activity of mt-HK was determined by a coupled assay according to Galina et al. (1999). Briefly, mt-HK activity was determined by NADH formation following the A$_{340}$ at 28°C. The assay medium contained: 20 mM Tris-HCl pH 7.4, 5 mM G6P, 6 mM MgCl$_2$, 1 mM β-NAD$^+$, 1 unit/mL G6PDH, 2 mM phosphoenolpyruvate, 0.1% (v/v) Triton X-100, and 10 units/mL pyruvate kinase. The reaction was started after adding 1 mM ATP. The final mitochondrial protein concentration varied from 0.05 to 0.1 mg/mL. When mt-HK activity was measured in intact PTM, Triton X-100 was omitted from the reaction medium that was in this case the same as the respiration buffer used to measure oxygen consumption.

G6PDH activity was assayed in a reaction medium containing 50 mM Tris-HCl buffer, pH 7.4, 6 mM MgCl$_2$, 0.1% (v/v) Triton X-100, and 0.5 mM β-NAD$^+$. The reaction was started by adding 1 mM G6P, and G6PDH activity was determined by measuring the absorption of β-NADPH formation at 340 nm. F$_6$F$_4$ATPase activity was determined by measuring the release of Pi from ATP in two different reaction media: (1) in the absence or (2) in the presence of 5 mM NaN$_3$. Both contained 20 mM Tris-HCl pH 8.0, 5 mM MgCl$_2$, 2 mM ATP, and 1 μM FCCP. The reaction was started by the addition of PTM protein and the difference between the activities in media 1 and 2 was considered as an authentic F$_6$F$_4$ATPase. The PTM protein concentration varied from 0.06 to 0.1 mg/mL.

The kinetic parameters were estimated by nonlinear regression analysis applied to the Michaelis-Menten equation using the program package supplied by Origin software (Galina et al., 1999).

Oxygen Uptake Measurements

Oxygen uptake was measured in an oximeter fitted with a water-jacket Clark-type electrode (Yellow Springs Instruments Co., model 5300) or in Oxytherm system for photosynthesis and respiration measurements in liquid phase (Hansatech Instruments). The PM (0.2 mg/mL) were incubated with 1 to 1.5 mL of the standard respiration buffer containing 0.5 mM mannitol, 10 mM Tris-HCl pH 7.2, 3 mM MgSO$_4$, 10 mM NaCl, 5 mM KH$_2$PO$_4$, 0.5 mM β-NAD$^+$, and 0.1% (v/v) faf-BSA. The cuvette was closed immediately before starting the experiments. Respiratory control ratio values were obtained with isolated PTM, after complex I inhibition by 1 μM rotenone and complex II activation by 10 μM succinate. Other additions are indicated in the figure legends.

ΔΨ$_{in}$ Determination

The ΔΨ$_{in}$ was measured by using the fluorescence signal of the cationic dye safranine O, which is accumulated and quenched inside energized mitochondria (Akerman and Wikström, 1997). PM (0.2 mg protein/mL) were incubated in the standard respiration buffer supplemented with 15 μM safranine. FCCP (2 μM) was used as a positive control to collapse ΔΨ$_{in}$. Fluorescence was detected with an excitation wavelength of 495 nm (slit 5 nm) and an emission wavelength of 586 nm (slit 5 nm) using a Hitachi model F-3010 spectrofluorimeter. Data were reported as arbitrary fluorescence units. Other additions are indicated in the figure legends.

Determination of Mitochondrial H$_2$O$_2$ Release

The H$_2$O$_2$ released from PTM was determined by the Ampex Red oxidation method, as previously described (Smith et al., 2004). Briefly, mitochondria (0.2 mg protein/mL) were incubated in the standard respiration buffer supplemented with 10 μM Ampex Red and 5 units/mL horseradish peroxidase. Fluorescence was monitored at excitation and emission wavelengths of 563 nm (slit 5 nm) and 587 nm (slit 5 nm), respectively. Calibration was performed by the addition of known quantities of H$_2$O$_2$.

Potato Tuber Slice Assays

Small square pieces were cut (8 mm diameter, 2 mm thickness) perpendicular to the stolon-apex axis of potato tuber (Tiessen et al., 2002). The tuber slices were taken from the middle of the tuber, avoiding the outer 3 mm and the tuber skin. The slices were incubated for 2 h in 10 mM MES-KOH, pH 6.5, in different conditions as shown in the figure legend. In oxygen consumption measurements we used 16 slices (0.3–0.45 g total slices) in a Hansatech oxygraph chamber of 1 mL and the oxygen consumption rate was monitored.
For \( \text{H}_2\text{O}_2 \) generation 20 slices were placed in 10 mL of the incubation buffer in an orbital shaker at 28°C. At different times, aliquots of 0.5 mL were removed and added to 1.5 mL respiration buffer containing 10 \( \mu \text{mol} \) Amplex Red and 5 units/mL horseradish peroxidase, and the fluorescence measured was taken as the amount of \( \text{H}_2\text{O}_2 \) accumulated in the incubation buffer of potato tuber slices.

**Protein Determination**

The protein concentration was determined as described by Lowry et al. (1951), using BSA as standard.

**Statistical Analysis**

Data were plotted with Origin 7.0 and analyzed by one-way ANOVA and a posteriori Tukey’s test. \( P < 0.05 \) were considered statistically different.

**ACKNOWLEDGMENTS**

We are grateful to Dr. Martha Sorensen for kind and valuable comments in improving the article. This work is dedicated to Leopoldo de Meis in honor of his 70th birthday.

Received September 3, 2008; accepted December 15, 2008; published December 24, 2008.

**LITERATURE CITED**


Dennis DT, Green TR (1975) Soluble and particulate glycocolysis in developing castor bean endosperm. Biochem Biophys Res Commun 64: 970–975


Miernyk JA, Dennis DT (1983) Mitochondrial, plastid, and cytosolic...
isozymes of hexokinase from developing endosperm of Ricinus communis. Arch Biochem Biophys 226: 438–468


