Evidence for Anion-translocating Plant Uncoupling Mitochondrial Protein in Potato Mitochondria*

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Transport properties of plant mitochondria from potato tubers were investigated using the swelling technique and membrane potential measurements. Proton-dependent swelling of fatty acid-depleted mitochondrial in potassium acetate with valinomycin was possible only in the presence of fatty acids (linoleic acid and 12-(4-azido-2-nitrophenylamino)dodecanoic acid), and was inhibited by various purine nucleotides including ATP, GDP, and GTP. Swelling representing uptake of hexanesulfonate was also inhibited by purine nucleotides. Also, the membrane potential of fatty acid-depleted potato mitochondria energized by succinate declined upon the addition of linoleic acid or 12-(4-azido-2-nitrophenylamino)dodecanoic acid, and this decrease was prevented by ATP and other purine nucleotides. These transport activities are identical to those reported for brown adipose tissue mitochondria and related to the uncoupling protein; therefore, we ascribed them to the plant mitochondrial uncoupling protein (PUMP). A major difference between plant and mammalian uncoupling protein is that PUMP transports small hydrophilic anions such as Cl− very slowly, if at all. We suggest that PUMP may play an important role in plant physiology, where a regulated uncoupling and thermogenesis can proceed during fruit and seed development.

Uncoupling of mitochondria is usually considered an unwanted pathological effect or an isolation artifact. For example, the beneficial effect of bovine serum albumin (BSA)1 on the coupling of plant mitochondria has been known for decades (1). Since plant tissues contain several lipases and hydrolases that, after cell breakage, cleave a significant amount of fatty acids (2), the effect of BSA, which removes fatty acids (FA), can be interpreted as prevention of FA-induced uncoupling. Thus, FA may represent a major part of the H+ leak in plant mitochondria (see Ref. 3), which were recently recognized to be essential for ATP supply to extrachloroplastic functions in photosynthetic cells even in light and for the important regulation of redox balance (4). However, regulated uncoupling could play an important physiological role in plant mitochondria, namely during fruit ripening, formation of seeds, flowering, seed dormancy, growth, and senescence, where a sudden or transient cutoff of efficient ATP production is required. Indeed, in all these events, FA metabolism plays a key role (2, 5, 6).

Long-chain FA are readily oxidized in most plant tissues by several pathways (2), including α- and β-oxidation in glyoxysomes and β-oxidation in mitochondria. In the latter, FA feed electrons to the respiratory chain and thus contribute to the production of ATP. However, studies of mitochondria in higher animals have shown that FA interfere with the energy-coupling system and act as weak uncouplers. FA thus play a dual role in cell energetics, being metabolites as well as regulators of oxidative phosphorylation efficiency (7–12). Recently, Skulachev (8) proposed the hypothesis of FA cycling, stating that some proteins allow for unipart of anionic FA, which can return in a protonated form across the bilayer. Hence, in this cycle, fatty acid behaves as a classic uncoupler. Indeed, up to now, at least two proteins have been shown to possess this property, the ADP/ATP carrier (8, 12) and the mitochondrial uncoupling protein (UcP), expressed specifically in brown adipose tissue of mammals (9–11). Surprisingly, a protein similar in all respects to UcP has been recently discovered by Vercesi et al. (13) in plant mitochondria. This protein was named plant uncoupling mitochondrial protein (PUMP) because it was found to mediate uncoupling in the presence of FA, and coupling was restored by ATP and BSA. Following a protocol for UcP isolation, it was possible to isolate from potato mitochondria a M, 32,000 protein. Both UcP and PUMP possess overall high hydrophobicity as reflected by their inability to be retained by hydroxyapatite, and both have identical M, values and function in the reconstructed system: purified reconstituted PUMP allowed for H+ transport in proteoliposomes, which was inhibited by ATP and GTP (13). However, interaction of FA with PUMP was not studied directly. Furthermore, the anion selectivity of PUMP, which has been characterized for UcP (14), has not been investigated. Our results confirm that PUMP transports protons in a FA-dependent manner and also catalyzes electrophoretic transport of hexanesulfonate. Unlike UcP, PUMP transports pyruvate and Cl− very slowly, if at all.

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1 The abbreviations used are: BSA, bovine serum albumin; FA, fatty acids; UcP, uncoupling protein; PUMP, plant uncoupling mitochondrial protein; MOPS, 3-(N-morpholino)propanesulfonic acid; FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone; AzDA, 12-(4-azido-2-nitrophenylamino)dodecanoic acid; PIMAC, plant mitochondrial inner membrane anion channel.
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EXPERIMENTAL PROCEDURES

**Chemicals and Biological Material**—Chemicals were mostly purchased from Sigma. 12-(4-Azido-2-nitrophenylamino)docosanoic acid was synthesized as described previously (10, 11). Potato tuber mitochondria were isolated from potatoes obtained from the local market essentially as described (15) but using a sucrose medium (16) containing 1% BSA, and mitochondria were also stored in this medium.

**Oxygen Uptake and Membrane Potential**—Oxygen uptake was measured with a Clarke-type oxygen probe (Yellow Springs Instrument Co.). Mitochondrial membrane potential ($\Delta \Psi$) was determined with 5 $\mu$m safranin (17) by measuring fluorescence at 586 nm (slit width of 5 nm) when excited at 495 nm (slit width of 5 nm) on a Hitachi Model F-4010 fluorometer. 1.5 ml of assay medium contained 125 mM sucrose, 65 mM KCl, 2.5 mM potassium P$_o$, 0.33 mM Na-EGTA, and 10 mM K-HEPES, pH 7.2. Routinely, 2 $\mu$m oligomycin, 33 $\mu$m propranolol, and 6 $\mu$m carboxyatractyloside were added to the medium if not indicated otherwise. 5 mM potassium succinate was used as a respiratory substrate.

**Swelling of Mitochondria**—This was measured essentially as described (10, 16, 18, 19) by detecting light scattering as indicated by the “absorbance” signal on an SLM DW2000 spectrophotometer (SLM-AMINCO, Urbana, IL). Media were 40% isoosmolar (when 270 mosmol is taken as 100%). For example, 55 mM potassium acetate, 5 mM Na-MOPS, 0.1 mM Tris/EDTA, and 0.2 mM Tris/EDTA, pH 7.2, and 55 mM sodium hexanesulfonate, 5 mM Na-MOPS, 0.1 mM Tris/EGTA, and 0.2 mM Tris/EDTA, pH 7.2, were used. EGTA and EDTA were omitted when MgCl$_2$ was added to study the effect of Mg$^{2+}$. Also media containing Cl$^-$ or pyruvate had similar compositions. 2.5 ml of each medium, routinely containing 200 $\mu$m propranolol and 1 $\mu$m antimycin A, was placed into a cuvette. In the case of potassium acetate, FA were added after 10 s, while 0.8 $\mu$m valinomycin was added at 15 s. In the case of sodium hexanesulfonate, 0.8 $\mu$m monensin and 0.8 $\mu$m FCCP were added at 10 s, in addition to valinomycin in other media. Since light scattering intensity is indirectly proportional to the mitochondrial volume and the reciprocal absorbance signal is directly proportional to the transport rates (19), we have taken transport rates as the slopes in plots of reciprocal absorbance versus time.

RESULTS

**Fatty Acids Induce H$^+$-dependent Swelling of Potato Mitochondria**—Potato tuber mitochondria thoroughly washed by several centrifugations with 10% BSA and stored in its presence exhibited only a negligible valinomycin-induced passive swelling in potassium acetate (Fig. 1A, trace a). One may interpret this as a lack of acetate uniport counterbalanced by K$^+$ uptake on valinomycin and a lack of H$^+$ efflux balanced by uptake of neutral acetic acid with the simultaneous K$^+$ uptake on valinomycin. However, the addition of a fatty acid, such as a model azido-FA, AzDA (Fig. 1A), or linoleic acid (Fig. 1B), which is naturally abundant in plants, induced a rapid swelling in potassium acetate with valinomycin. This transport was routinely containing 200 $\mu$m propranolol and 1 $\mu$m antimycin A, was placed into a cuvette. In the case of potassium acetate, FA were added after 10 s, while 0.8 $\mu$m valinomycin was added at 15 s. In the case of sodium hexanesulfonate, 0.8 $\mu$m monensin and 0.8 $\mu$m FCCP were added at 10 s, in addition to valinomycin in other media. Since light scattering intensity is indirectly proportional to the mitochondrial volume and the reciprocal absorbance signal is directly proportional to the transport rates (19), we have taken transport rates as the slopes in plots of reciprocal absorbance versus time.

**Hexanesulfonate Is Transported by PUMP**—Since UcP, the mammalian prototype of PUMP, translocates several classes of monovalent unipolar anions (14), we tested hexanesulfonate, as a representative anion, to investigate whether it is also transported by PUMP. Indeed, we observed swelling dependent on the simultaneous presence of both monensin and FCCP in the sodium salt of hexanesulfonate in the presence of propranolol (Fig. 3). Monensin ensures a Na$^+/H^+$ exchange, and FCCP...
counterbalances the $H^+$ efflux.2 Simultaneous uptake of $Na^+$ and hexanesulfonate anion is then reflected by mitochondrial swelling. Propranolol was used to exclude the PIMAC-mediated hexanesulfonate uniport since alkylsulfonates were also found to be substrates of the inner membrane anion channel (14). As in the case of FA-induced $H^+$ transport, hexanesulfonate uptake was completely inhibited by 3 mM ATP (Fig. 3, upper trace) and by GDP and GTP (data not shown). Moreover, similar to UcP (9), the affinity of FA for PUMP seems to be higher than the affinity of hexanesulfonate, which might interact with PUMP in the same domain, as suggested, for example, by 35% inhibition of the hexanesulfonate uptake by 20 $\mu$M azido-FA (Fig. 3, middle trace).

**PUMP Does Not Translocate $Cl^-$ and Pyruvate—**Mammalian UcP translocates $Cl^-$ (20, 21) and other monovalent anions such as alkylsulfonates, oxohalogenides, and monovalent phosphate analogs (14) and pyruvate.3 As observed previously (16), potato tuber mitochondria swell in KCl medium with valinomycin, but this transport was fully (by 98%) prevented by propranolol (Fig. 4), an inhibitor of the inner membrane anion channel. The residual rate of $Cl^-$ uniport was nearly identical (110%) to the basal swelling rate in potassium acetate with BSA (Fig. 1B, trace d) and accounted for <10% of the maximum rate of FA-induced $H^+$ transport (Fig. 2) and hexanesulfonate uniport (Fig. 3). Since the inner membrane anion channel is not

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2 Probably because of the existence of the sodium-selective $Na^+/H^+$ exchanger, a slow $Na^+/H^+$ antiport was observed even in the absence of monensin in the presence of FCCP, but these rates were as low as those in the presence of 3 mM ATP with monensin and FCCP. Rates obtained with monensin and without FCCP reached <18% of the rates with monensin and FCCP. This indicates the absence of a hexanesulfonate/$H^+$ symporter.

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910 μM, respectively, at pH 7.2. The $K_i$ values for GDP and GTP (data not shown) were similar high (~1 mM). As expected, the addition of MgCl$_2$ further shifted the $K_i$ to much higher values (data not shown), so that even at 1 mM ATP, no inhibition occurred. This effect, when Mg$^{2+}$ chelates ATP and turns it into a non-interacting complex, was also observed in the case of UcP.

Energy Coupling—Both linoleic acid (Fig. 6) and AzDA exerted an uncoupling effect on the energetics of potato tuber mitochondria as manifested by increasing the resting state respiration with succinate in the presence of oligomycin, propranolol, and carboxyatractyloside. 0.5 mM ATP inhibited this increase by 50%. Cyanide blocked respiration independently of FA addition, indicating the absence of the alternative oxidase pathway (1) under these conditions. In a parallel experiment, upon the addition of 24 μM linoleic acid, the membrane potential ($\Delta\Psi$) decreased instantaneously to very low values (Fig. 7, trace e). With increasing ATP concentration, the $\Delta\Psi$ decline was smaller (Fig. 7, traces b–d). Thus, ATP prevented the FA-induced uncoupling. An identical pattern of FA-induced uncoupling was found in mitochondria isolated from red tomatoes (data not shown). Unlike in mammalian mitochondria, for example, in heart (22), the uncoupling effect of FA in potato tuber mitochondria is not prevented by carboxyatractyloside (see also Ref. 23), an inhibitor of the ADP/ATP carrier. The FA effect always contained a component insensitive to ATP, which is observed in all types of mitochondria (8, 22). We have verified this with rat liver mitochondria (data not shown). Also, high Mg$^{2+}$ concentration did not have any effect, contrary to pea stem mitochondria (23).

A more complicated situation, however, was found at low (2.7 μM) linoleic acid concentration. Its addition slightly accelerated respiration, but only in the presence of ATP. Surprisingly, in the absence of ATP, the low FA dose inhibited respiration. The reason for this and for the lack of such inhibition at higher FA concentrations is not clear at present. Nevertheless, this phenomenon led to the observation of a biphasic $\Delta\Psi$ decline (data not shown), where the second phase with progressively falling $\Delta\Psi$ occurred because of inhibition of respiration. Two effects of ATP were then superimposed, release of respiratory inhibition and prevention of FA-induced uncoupling. 0.5 mM ATP then inhibited completely the uncoupling induced by 2.7 μM linoleic acid.
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FIG. 7. Membrane potential changes during fatty acid-induced uncoupling of potato tuber mitochondria respiring with succinate. Changes in membrane potential (ΔΨ), induced by 24 μM linoleic acid, monitored fluorometrically by safranin in a parallel experiment to that described in the legend to Fig. 6 are shown. Potato tuber mitochondria were pretreated with 1% BSA and resuspended in a medium containing succinate as a respiratory substrate and 333 μM propranolol, 10 μM atractyloside, 1 μM oligomycin, and 5 μM safranin. They reached the highest coupled state instantaneously upon resuspension (as indicated by minimum fluorescence). The potential was stable, as documented by trace a, when no FA was added, but 1 μM FCCP added after 4 min caused a fast and complete uncoupling. Trace e shows the rapid and complete uncoupling induced by linoleic acid in the absence of ATP. Traces b–d represent an intermediate uncoupling by 24 μM linoleic acid in the presence of 0.5, 0.2, and 0.1 mM ATP, respectively.

DISCUSSION

The existence of PUMP (13), a specialized protein in plant mitochondria ensuring regulated uncoupling, is surprising and could facilitate a better understanding of some physiological events in plants. Our data demonstrate important similarities in the function of PUMP and mammalian UcP, which is specifically located in brown adipose tissue. Similarity in their structures must be further verified by cloning and sequencing of PUMP cDNA. The existence of phylogenetically distant, yet similarly functional transport proteins such as PUMP and UcP can help us to better understand the evolution of transport proteins and consequently also the mechanisms of their function. Up to now, several cases have been revealed in plants (13, 23) as well as in higher animals (8–12, 22) in which FA, via proteins and consequently also the mechanisms of their function. Up to now, several cases have been revealed in plants (13, 23) as well as in higher animals (8–12, 22) in which FA, via interaction with specific mitochondrial membrane proteins, enable the regulation of mitochondrial uncoupling. Such uncoupling should not be regarded as a mere shutting between the two limiting states, fully coupled and fully uncoupled. Uncoupling might serve as well to optimize the efficiency of oxidative phosphorylation under different metabolic and physiological regimes (24). We might speculate that the termination of all synthetic processes during the final stages of seed formation and during senescence can be regulated via the PUMP-mediated uncoupling of mitochondria by FA. Especially associated with fruit ripening is the well known phenomenon of respiration burst in climacteric fruits (6, 25, 26). We hypothesize that it can be attributed to the PUMP-mediated uncoupling of mitochondria because PUMP has been found also in fruits, namely in tomatoes and avocados, and because PUMP, being a protonmotive force (Δp) consumer, can regulate Δp directly and much more effectively than numerous regulatory mechanisms switching to the nonphosphorylating routes to molecular oxygen. Such mechanisms are provided by alternative oxidase (1, 27–29), external NAD(P)H dehydrogenase, and internal rotenone-insensitive NAD(P)H dehydrogenase (30). On the contrary, during seed germination, de novo synthesis of mitochondria should not be required in the initial stages, but some hormonal effects, analogous to norepinephrine stimulation of thermogenesis (20), might restore the coupled state via blockage of PUMP.

Our results support all of the above considerations since they show that PUMP is a transport protein that interacts with FA, which leads to uncoupling. Because of its similarity to UcP, for which a mechanism of fatty acid cycling has recently been proposed (9), we also suggest that FA anions are translocated by PUMP. The phenomenon of swelling of potato mitochondria in potassium acetate with valinomycin induced by FA is then interpreted as illustrated in Fig. 8. We have also shown that PUMP enables uniport of hexanesulfonate, which most probably shares the same transport pathway with FA. In the case of UcP, this has been demonstrated by many kinetic and competition experiments (9–11, 18, 31). For PUMP, we have shown in this paper that AzDA can inhibit hexanesulfonate transport (Fig. 3). Another similarity between PUMP and UcP is their interaction with purine nucleotides. Although PUMP seems to possess rather a lower affinity for nucleotides in comparison with UcP and concentrations of several millimolar are required to block its transport completely, just this allows for its role at normal physiological ATP concentrations. Actually, free Mg2+ may adjust the inhibitory ability of ATP so that PUMP can uncouple mitochondria in the presence of 1 mM ATP. In turn, PUMP would be inhibited at the elevated ATP levels, similar to UcP (32). We suggest that both nucleotide and Mg2+ levels in plant cells could play the role of a third messenger in hormonal

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regulation of FA-mediated uncoupling (second messenger being Ca\(^{2+}\) or other signaling). Nevertheless, we have shown (Figs. 6 and 7) that, unlike in brown fat mitochondria (20), ATP is able to restore the highest coupled state in the presence of FA.

The lower Cl\(^{-}\) permeability through PUMP than through mammalian UcP may be attributed to higher hydrophobicity of a presumably internal\(^5\) translocation binding site when compared with UcP, which enables such a transport. Nevertheless, even UcP has a 10,000 times higher affinity for lauric acid and undecanesulfonate compared with its affinity for Cl\(^{-}\) (9). However, the \(V_{\text{max}}\) for Cl\(^{-}\) uniport via UcP reaches 40% of the \(V_{\text{max}}\) for laurate uniport (9). Our data indicate that if one subtracts a basal swelling with BSA, the net Cl\(^{-}\) uniport rates would account for only 1% of linoleate transport. Thus, if PUMP and UcP, which enable such a transport, nevertheless, would translocate Cl\(^{-}\), the \(V_{\text{max}}\) should be <1% of the \(V_{\text{max}}\) for linoleate transport. Such inability of PUMP to translocate anions that should statistically “bombard” the membrane in order to reach the internal binding site can be a result of a tighter interaction of PUMP with surrounding mitochondrial lipids. Phylogenetically, PUMP could have lost or not developed this ability since, in plant contrary to mammalian mitochondria, PIMAC, insensitive to matrix Mg\(^{2+}\), ensures Cl\(^{-}\) transport under normal conditions (16).

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\(^{5}\) There are numerous lines of evidence for intramembrane location of the anion translocation binding site in UcP (9, 14, 32), namely non-existence of nontransportable substrate analogs; increase in transport rate, \(V_{\text{max}}\), and affinity (decrease in \(K_{\text{m}}\)) with increasing hydrophobicity of substrates; shape of flux-voltage characteristics, etc.

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