Alteration of Plant Mitochondrial Proton Conductance by Free Fatty Acids

UNCOUPLING PROTEIN INVOLVEMENT*

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We characterized the uncoupling activity of the plant uncoupling protein from Solanum tuberosum (StUCP) using mitochondria from intact potato tubers or from yeast (Saccharomyces cerevisiae) expressing the StUCP gene. Compared with mitochondria from transfected yeast, StUCP is present at very low levels in intact potato mitochondrial membranes (at least thirty times lower) as shown by immunodetection with anti-UCP1 antibodies. Under conditions that ruled out undesirable effects of nucleotides and free fatty acids on uncoupling activity measurement in plant mitochondria, the linoleic acid-induced depolarization in potato mitochondria was insensitive to the nucleotides ATP, GTP, or GDP. In addition, sensitivity to linoleic acid was similar in potato and in control yeast mitochondria, suggesting that uncoupling occurring in potato mitochondria was because of a UCP-independent proton diffusion process. By contrast, yeast mitochondria expressing StUCP exhibited a higher sensitivity to free fatty acids than those from the control yeast and especially a marked proton conductance in the presence of low amounts of linoleic acid. However, this fatty acid-induced uncoupling was also insensitive to nucleotides. Altogether, these results suggest that uncoupling of oxidative phosphorylation and heat production cannot be the dominant feature of StUCP expressed in native potato tissues. However, it could play a role in preventing reactive oxygen species production as proposed for mammalian UCP2 and UCP3.

The discovery in plants of a mitochondrial uncoupling protein resembling uncoupling proteins from mammals raised the possibility of a new uncoupling mechanism involving a transport pathway for backflow of protons into the matrix of plant mitochondria (1, 2). Indeed, a protein fraction containing a purine nucleotide-sensitive, free fatty acid-activated uncoupling activity, called PUMP, has been isolated from potato mitochondria and characterized after reconstitution in proteoliposomes (1). In addition, various cDNAs coding for putative UCPs were identified in plants such as potato, Arabidopsis thaliana, and skunk cabbage (Symplocarpus foetidus), a thermogenic plant from the Aracea family (3–6). Recently, the Arabidopsis thaliana AtUCP1 gene was expressed in Escherichia coli and the isolated protein reconstituted into liposomes (7). Like mammalian UCPs, the plant UCP1 requires free fatty acids for its activity and is inhibited by purine nucleotides (GDP, ATP, GTP, or ADP) (7–10). At the mitochondrial level, it has been proposed that ATP, GTP, or GDP were absolutely required to get a fully coupled state in potato tuber mitochondria oxidizing succinate (1, 8). Similarly, it has been suggested that the fatty acid-induced uncoupling in wheat mitochondria was sensitive to purine nucleotides (11).

In this paper, we sought to characterize better the properties of plant UCP-like protein by comparing the fatty acid-dependent uncoupling capacity of either native UCP present in intact potato mitochondria or StUCP expressed at a high level in yeast mitochondria. It is shown that StUCP increases the proton conductance in yeast expressing StUCP but not in native mitochondrial membranes in which the protein is present at a too low level. Similarities in behavior and properties between StUCP expressed in yeast and PUMP are discussed.

MATERIALS AND METHODS

Chemical and Biological Materials—Chemicals were from Sigma unless otherwise indicated. Potato tubers (Solanum tuberosum L., cv Bintje) were from a local producer and kept at 4 °C at least for 1 week before use.

Isolation of Plant Mitochondria—Potato tuber mitochondria were isolated using a classical procedure and purified on Percoll density gradient containing 0.1% BSA (12). After washing in a medium containing 300 mM mannitol, 10 mM MOPS-KOH (pH 7.2), 1 mM EDTA, and 0.01% BSA (fatty acid-free), they were resuspended in a minimum volume of the above medium with or without 0.01% BSA.

Isolation of Yeast Mitochondria—Yeast were grown at 30 °C in liquid basal medium (1.7% yeast nitrogen base, 0.5% (NH4)2SO4, 0.1% CSM-urea, 0.1% KH2PO4, 20 mg/liter tryptophan, 40 mg/liter adenine, pH 4.5, 2% lactate, 0.1% glucose) until 4–5 A units. The culture was then diluted to a final absorbance of 0.3 units and either grown overnight (control cells) before harvesting or grown for 2 h before inducing UCP expression by adding 2% galactose to the medium and then growing overnight before harvesting. Spheroplasts were obtained from 10 g of yeast cells by digestion in the presence of 1.2 mM β-mercaptoethanol with 50 mg of Zymolase in 100 ml of digestion buffer (1.2 mM sorbitol, 1 mM MgCl2, 0.1 M Tris, pH 7.0, 0.1% BSA, 10 mM MgSO4, 200 U/ml Zymolase, 50 µg/ml ampicillin, 50 µg/ml kanamycin, 50 µg/ml chloramphenicol).

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5 The abbreviations used are: UCP, uncoupling protein; StUCP, uncoupling protein from Solanum tuberosum; BSA, bovine serum albumin; MOPS, 3-(N-morpholino)propanesulfonic acid; TPP++, tetraphenylphosphonium; LA, linoleic acid.
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mm EDTA, 75 mM phosphate buffer, pH 7.5) at 30°C. Zymolase was eliminated by two washes in digestion buffer and the final pellet was resuspended in 100 ml of mitochondria buffer (0.6 M sorbitol, 4 mM EGTA, 75 mM phosphate buffer, pH 6.8, 0.2% BSA). Spheroplasts were then gently lysed by mechanical shearing and mitochondria were purified by differential centrifugations and washed in mitochondria buffer containing only 0.01% BSA (13).

Membrane Potential and Oxygen Consumption—Membrane potential was measured continuously and simultaneously to oxygen consumption using a tetraphenylphosphonium (TPP⁺)-sensitive electrode in the presence of 4 μM TPP⁺ and a Clark-type oxygen electrode at 20°C (14). The membrane potential values were calculated using the equation of Kamo et al. (15) without corrections for cation binding and assuming a mitochondrial volume of 1 μl/mg of protein. Air-saturated electrode medium contained: 0.3 mM mannitol, 10 mM phosphate buffer, 10 mM MOPS, 5 mM KCl, and 5 mM MgCl₂, pH 7.2, for potato mitochondria and 0.65 mM mannitol, 20 mM MOPS, 0.5 mM EGTA, 10 mM K₂HPO₄, and 2 mM MgCl₂, pH 6.8, in the case of yeast mitochondria (16). Routinely, 2 μM oligomycin, 6 μM carboxyatractyslide and nigericin (400 ng/ml of protein) were added to the medium if not indicated otherwise, and 5 mM succinate (in the presence of 10 μM rotenone) or 1 mM external NADH were used as substrate. For potato mitochondria, measurements were performed in the absence or presence of BSA (0.01 or 0.1%). Unless otherwise indicated, measurements on yeast mitochondria were carried out in the presence of 0.01% BSA (1.6 μg/ml) as described in Ref. 15. Proton conductance curves were determined as the plots of oxidation rates versus membrane potential in the resting state (with either 5 mM succinate or 1 mM NADH as substrate) using increasing concentrations of cyanide (ranging between 0 and 1 mM) to progressively inhibit the respiratory chain.

Mitochondrial membrane potential changes were separately monitored at 20°C with 5 μM safranine O by measuring fluorescence at 586 nm when excited at 495 nm (slit widths set at 5 nm) on a PerkinElmer fluorimeter. The standard medium (2 ml) contained: 0.25 mM of mitochondria, 0.125 mM sucrose, 65 mM KCl, 0.33 mM EGTA, 1 mM MgCl₂, 2.5 mM KH₂PO₄, and 10 mM Hepes, pH 7.2 (15). Routinely, 2 μM oligomycin, 6 μM carboxyatractyslide and nigericin (400 ng/ml of protein) were added.

Expression of Potato Uncoupling Protein in Yeast—The StUCP coding sequence was introduced into recombinant expression vectors previously used for UCP1 (13) and UCP2 (17). Integrity of all expression vectors was checked by PCR using specific UCP1 primers and diploid yeast (Saccharomyces cerevisiae) strain W303 was transformed with these modified vectors using the thermal shock method described by Chen et al. (18). Expression of StUCP in mitochondrial membranes was checked by Western blot analysis using anti-UCP1 antibodies (19).

Immunological Detection of UCP—After electrophoretic separation on 11% polyacrylamide gels under denaturing conditions (SDS-PAGE), proteins were electrotransferred for 30 min at 135 mA on a 0.2-μm nitrocellulose membrane (Bio-Rad) using a Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad) system and Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3). Detection of plant UCP was performed with affinity purified anti-UCP1 antibodies, referred to as 375.5 (19), diluted 4000-fold and horseradish peroxidase linked anti-sheep IgGs as secondary antibodies according to the manufacturers recommendations for the Western blot chemiluminescence detection system (Renaissance Kit from PerkinElmer Life Sciences). Quantification was performed with ImageQuant software.

RESULTS

Immunological Characterization of StUCP and Detection Limits—To characterize UCP in potato tuber mitochondria, we used an antibody raised against mammalian UCP1 that was first tested for its ability to recognize specifically the 32-kDa potato UCP (StUCP) expressed in yeast. Fig. 1A shows a specific hybridization with mitochondria from yeasts that express the StUCP cDNA (lane 1), or from control yeasts that were not induced for StUCP (lane 2). The hybridized band exhibited a hybridization with the anti-UCP1 antibodies previously incubated with increasing amounts of competitor. The anti-UCP1 antibodies was obtained when 40 μg of total mitochondrial protein as competitor (Fig. 1B): a progressive extinction occurred with increasing amounts of competitor. The anti-UCP1 antibodies used in this study recognized A. thaliana UCP (AtUCP) expressed in yeast as well, indicating that they are good tools for UCP detection in plants (data not shown). With potato mitochondria, a faint but significant hybridization with anti-UCP1 antibodies was obtained when 40 μg of total mitochondrial protein were loaded (Fig. 1C, lane 3), indicating the presence of UCP-like protein in potato mitochondria as reported in the literature (20). However, 5 μg of mitochondrial protein from yeast expressing StUCP (lane 2) were sufficient to show an important signal, whereas control yeast showed a total absence of detection under these conditions (lanes 3 and 6). This result clearly indicates the presence of a very low level of StUCP protein in potato tuber mitochondria (about 30 times less than in yeast expressing StUCP).

Effect of Nucleotides on Potato Mitochondria in Resting State—UCP-like protein found in potato mitochondria has been supposed to function similarly to the uncoupling protein of brown adipose tissue in mediating a fatty acid-dependent proton leak sensitive to nucleotides (especially ATP) (8–10). However, ATP exerts several effects on mitochondria oxidizing succinate in the resting state: activation/deactivation of succinate dehydrogenase (21, 22), activation of F₄₂₃-ATPase (23). We therefore investigated the effects of ATP on nonphosphorylating respiration rate, membrane potential, and proton conductance in potato mitochondria using two different respiratory substrates (succinate and external NADH) in the presence of inhibitors of ATP synthase and ADP/ATP carrier (oligomycin and atractylloside, respectively). As shown in Fig. 2A, trace 1). By contrast, the value of membrane potential triggered by addition of external NADH immediately reached the same value as that obtained with succinate + ATP, and subsequent addition of 1 mM ATP had no effect on membrane potential. Similar effects were obtained with ATP concentrations ranging between 200 μM and 2 mM (data not shown). These data indicate that ATP was not required to
obtain fully coupled mitochondria but rather that ATP led to succinate dehydrogenase activation. When experiments were carried out in the absence of BSA in the respiration medium, values of membrane potential in the resting state were lower because of the presence of endogenous free fatty acids by BSA led to a shift of the conductance curve toward higher values of membrane potential. This provides evidence that ATP increased both succinate oxidation and membrane potential. Thus, we have studied the linoleic acid-induced uncoupling activity in potato mitochondria. Either succinate or external NADH was used as respiratory substrate.

**Fig. 2. Effect of ATP on nonphosphorylating respiration, membrane potential, and proton conductance of potato tuber mitochondria.** A, 0.35 mg of mitochondria depleted of free fatty acids oxidizing 5 mM succinate (trace 1) or 1 mM external NADH (trace 2) in respiration medium containing 0.1% BSA. 1 mM ATP was added in the steady state, then membrane potential was collapsed with 0.2 mM valinomycin. B, 0.35 mg of mitochondria containing endogenous fatty acids oxidizing 10 mM succinate (trace 1) or 1 mM external NADH (trace 2) in respiration medium without BSA. 1 mM ATP, 0.1% BSA, and 0.2 mM valinomycin were successively added. C, proton conductance curves were determined as the plots of succinate oxidation rate versus membrane potential in the resting state on mitochondria containing endogenous fatty acids (see “Materials and Methods”), in the absence of ATP (empty squares) or presence of 1 mM ATP (full circles), or in the presence of 1 mM ATP plus 0.1% BSA (full triangles).

Taken together, these results suggest that the ATP-induced change in succinate oxidation was not compatible with increased coupling in potato mitochondria.

**Effect of Linoleic Acid (LA) and Nucleotides on Potato Mitochondria—**If plant uncoupling protein had to function as the uncoupling protein of brown adipose tissue, its activity should be stimulated by addition of exogenous free fatty acids and inhibited by nucleotides such as ATP, GTP, or GDP (8–10). Thus, we have studied the linoleic acid-induced uncoupling activity in potato mitochondria in the nonphosphorylating state. Either succinate or external NADH was used as respiratory substrate and safranine as membrane potential probe (as described under “Materials and Methods”).

In A and B, safranine fluorescence was triggered by adding mitochondria (0.25 mg of protein) to 2 ml of respiring medium without BSA containing either 5 mM succinate (A) or 1 mM external NADH (B). In both cases measurements were carried out in the absence (continuous line 1) or presence (dotted line 2) of nigericin (mg, 400 ng/mg protein). After addition of 16 mM LA, 1 mM GTP (or GDP) and/or 1 mM ATP were successively added. Further additions were: 0.1% BSA and 0.5 mM KCN. In C and D, simultaneous measurements of oxidation rate and membrane potential with the TPP$^+$ electrode on mitochondria oxidizing 5 mM succinate (C) or 1 mM NADH (D). In 1 ml of respiration medium without BSA containing mitochondria (0.35 mg of protein), 10 mM LA, 1 mM NTP (either ATP for trace 1 or GTP for trace 2), 0.1% BSA, and 0.2 mM valinomycin were added successively. D corresponds to the same recording as C except for respiration substrate.
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caused an immediate release of safranine (increase of fluorescence) that was followed by a slow re-uptake of the probe (decrease of fluorescence). This effect was totally abolished in the presence of nigericin (an ionophore that exchanges \( \mathrm{H}^+ \) and \( \mathrm{K}^- \)), suggesting that safranine re-uptake was due either to a change in transmembrane \( \Delta \mathrm{pH} \) and/or to mitochondrial swelling triggered by LA itself as previously evidenced (8). Because this effect was also detectable with the TPP\(^{\pm} \) electrode, nigericin was included in all further experiments (Fig. 3, C and D). To test UCP involvement in the LA-induced depolarization, ATP, GTP, or GDP were added to mitochondria to inhibit the UCP-dependent proton leak. Whatever the substrate (succinate or NADH), GTP (1 mM) (or GDP, data not shown) did not exhibit a re-coupling effect (Fig. 3, C and D). Only ATP was able to repolarize mitochondria when succinate was present and this repolarization was associated with an increase in oxidation rate (Fig. 3, A and C). With external NADH, no nucleotide-dependent repolarization of mitochondria could be detected (Fig. 3, B and D). Thus, either StUCP was involved in the LA-induced depolarization of potato mitochondria as previously suggested (8, 10), but it had to be insensitive to nucleotides, or this uncoupling is UCP-independent and unspecific.

**StUCP-dependent Uncoupling When Expressed in Yeast**—To elucidate the role of plant UCP in uncoupling of potato mitochondria, StUCP was expressed at a high level in yeast and the effects of LA and nucleotides on isolated mitochondria respiring on external NADH in the nonphosphorylating state were studied. Experiments carried out in the absence of exogenous free fatty acid (in the presence of 16 \( \mu \text{M} \) BSA) showed that membrane potential and respiratory rate were similar in yeast mitochondria expressing StUCP and controls (212 ± 2 and 216 ± 2 mV for \( \Delta \psi \), respectively, and 193 ± 20 and 154 ± 15 nmol of \( \mathrm{O}_2 \)-min\(^{-1}\)-mg\(^{-1}\) protein for respiration, respectively). As a consequence, the proton conductance in the resting state (assuming an \( \mathrm{H}^+/\mathrm{O}_2 \) ratio of 12 for external NADH oxidation) was comparable in the two cases (10.9 ± 0.75 and 8.7 ± 0.35 nmol \( \mathrm{H}^+\)-min\(^{-1}\)-mg\(^{-1}\)-protein mV\(^{-1}\)).

Uncoupling of yeast mitochondria by exogenous free fatty acid under the conditions described in Ref. 16 is shown in Fig 4. For LA concentrations ranging between 2 and 10 \( \mu \text{M} \) (corresponding to a molar LA:albumin ratio between 1.2 and 6), the oxidation rate was significantly enhanced in StUCP mitochondria while no changes occurred in control yeast or in potato mitochondria (Fig. 4A). In the same range of LA concentrations, a progressive depolarization occurred in StUCP mitochondria while no or little change was found in control yeast or potato mitochondria (Fig. 4B). Interestingly, for higher LA concentrations, oxidation rates increased in parallel in the three categories of mitochondria. Because in control yeast mitochondria, the LA-dependent respiration and depolarization reflected a UCP-independent uncoupling process, we can thus assume that StUCP expressed in yeast was responsible for an uncoupling that required the lowest LA concentrations. Under these conditions, a maximal UCP-dependent uncoupling corresponding to a depolarization of about 35 mV and enhancement in oxidation rate of ~40% corresponding to 80 nmol/min\(^{-1}\)-mg\(^{-1}\) protein was obtained in the presence of about 10 \( \mu \text{M} \) LA.

To assess the effect of nucleotides on LA-induced activity of plant uncoupling protein expressed in yeast, the flux-force relationships were established in the presence of 7.5 \( \mu \text{M} \) LA (molar ratio LA:albumin of 4.5) to activate StUCP and in the presence or absence of nucleotides. As shown in Fig. 4C, respiration and membrane potential were slightly inhibited by ATP, GTP, or GDP (1 mM), suggesting that nucleotides affected the respiration without directly affecting proton leaks. Indeed, proton conductance curves established in the presence of nucleotides clearly showed that changes in \( \Delta \psi \) followed changes in respiration and that the relationships between oxidation rate and membrane potential were identical (same conductance curves). By contrast, removal of added LA by 0.1% BSA led to a shift of proton conductance curve toward higher values of membrane potential (i.e., lower values of proton conductance). This result suggests that StUCP expressed in yeast mediated proton leak and mitochondrial uncoupling, which were insensitive to nucleotides.

**Fig. 4.** Plant UCP (StUCP)-dependent mitochondrial uncoupling activated by linoleic acid in mitochondria from yeast expressing StUCP, from control yeast, or from potato tuber. Experimental conditions are described under "Materials and Methods." A, oxidation rate in the resting state in the presence of 0.01% BSA (1.6 \( \mu \text{M} \)) and 1 mM NADH as substrate. B, membrane depolarization under the same conditions. C, effect of 1 mM ATP, GTP, or GDP on membrane proton leak of StUCP yeast mitochondria (in the presence of 7.5 \( \mu \text{M} \) LA, to activate UCP) as determined by the flux-force relationship between oxidation rate and membrane potential, in the absence of nucleotides (full circles) or in the presence of 1 mM ATP (full triangles), 1 mM GDP (empty squares), 1 mM GTP (crosses). Effect of 0.1% BSA on membrane proton leak (open triangles).
To characterize the functional aspects of a plant uncoupling protein (StUCP), the gene encoding this protein was expressed in S. cerevisiae and the level of peptide found in the mitochondria was probed using antimammalian UCP1 antibodies. In the yeast system, the level of expression of UCP was similar between StUCP and UCP1. Based on data reported for UCP1 (27), the amount of StUCP is about 10% of the total mitochondrial protein. We showed that StUCP yeast mitochondria exhibited a diminished resting potential and a high sensitivity to uncoupling by LA. The StUCP-dependent uncoupling activity measured in yeast (expressed as LA-stimulated oxidation rate) was similar in mitochondria from yeast expressing StUCP (this work) and mammalian UCP2 (16), namely about 80 nmol of \( O_2 \) min \(^{-1} \) mg \(^{-1} \) protein or 1 \( \mu \)mol of \( H^+ \) min \(^{-1} \) mg \(^{-1} \) protein, assuming a \( H^+/O_2 \) ratio of 12 for external NADH oxidation.

Because StUCP represented at most 10% of the total mitochondrial protein, the minimal proton transport capacity of StUCP could be estimated as \( \sim 10 \) \( \mu \)mol of \( H^+ \) min \(^{-1} \) mg \(^{-1} \) UCP protein, under these conditions. With the exception of UCP1 (28), this value is consistent with those reported for all mammalian UCPs (from 8 to 30 \( \mu \)mol of \( H^+ \) min \(^{-1} \) mg \(^{-1} \) UCP protein) when expressed in E. coli and reconstituted in proteoliposomes (7, 29). StUCP protonophoric activity in yeast mitochondria is also in agreement with the ability of plant UCP reconstituted in proteoliposomes to collapse the proton gradient, as evidenced with AtUCP1 expressed in E. coli (7) or with the PUMP fraction isolated from potato mitochondria (1, 30). However, in the case of PUMP it is difficult to make a precise comparison because the amino acid sequence of PUMP is still unknown. In our experiments we assayed the uncoupling activity of the StUCP peptide in recombinant yeast, whereas Verseci et al. (1) found a protonophoric activity after reconstitution in proteoliposomes of a hydroxyapatite column fraction. Therefore it is unclear whether both uncoupling activities are related to the same protein, i.e., whether StUCP and PUMP are the same peptide. If they are, then the nucleotide-binding properties of StUCP are somehow different in liposomes and in yeast mitochondria. StUCP would be inhibited by nucleotides in liposomes but not in yeast mitochondria. The protein contains the sequence motifs for nucleotide sensitivity (5, 31, 32) and a low nucleotide sensitivity (inhibition constant between 0.8 and 1.7 mM) was found for AtUCP1 reconstituted from E. coli (7). As proposed for mammalian UCP2 or UCP3 (16, 28, 33), potato UCP might be relevant. Under appropriate conditions (namely, external uncoupling protein when it is expressed in large amounts but it is not present in mammalian UCP2 or UCP3, uncoupling of oxidative phosphorylation and heat production are not the dominant features. A role in preventing reactive oxygen species production has been proposed (24) and was recently demonstrated for UCP2 and UCP3 using knockout mice (35, 36). It has been suggested that reactive oxygen species could directly activate the UCPs (37) and that this mild uncoupling of mitochondrial respiration could, in turn, reduce the production of reactive oxygen species. However, such a reactive oxygen species that induced mild uncoupling has not been confirmed for UCP2 (38) and this should be further investigated in the case of the StUCP.

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