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Activation and Function of Mitochondrial Uncoupling Protein in Plants*

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Plant mitochondrial uncoupling protein (UCP) is activated by superoxide suggesting that it may function to minimize mitochondrial reactive oxygen species (ROS) formation. However, the precise mechanism of superoxide activation and the exact function of UCP in plants are not known. We demonstrate that 4-hydroxy-2-nonenal (HNE), a product of lipid peroxidation, and a structurally related compound, *trans*-retinal, stimulate a proton conductance in potato mitochondria that is inhibitable by GTP (a characteristic of UCP). Proof that the effects of HNE and *trans*-retinal are mediated by UCP is provided by examination of proton conductance in transgenic plants overexpressing UCP. These experiments demonstrate that the mechanism of activation of UCP is conserved between animals and plants and imply a conservation of function. Mitochondria from transgenic plants overexpressing UCP were further studied to provide insight into function. Experimental conditions were designed to mimic a bioenergetic state that might be found *in vivo* (mitochondria were supplied with pyruvate as well as tricarboxylic cycle acids at *in vivo* cytosolic concentrations and an exogenous ATP sink was established). Under such conditions, an increase in UCP protein content resulted in a modest but significant decrease in the rate of superoxide production. In addition, ^{13}C-labeling experiments revealed an increase in the conversion of pyruvate to citrate as a result of increased UCP protein content. These results demonstrate that under simulated *in vivo* conditions, UCP is active and suggest that UCP may influence not only mitochondrial ROS production but also tricarboxylic acid cycle flux.

Mitochondrial uncoupling proteins are integral membrane proteins that reside in the inner mitochondrial membrane and belong to a large family of mitochondrial anion carriers (1). They catalyze a proton conductance that results in the dissipation of the proton gradient across the inner mitochondrial membrane. As such, they partially uncouple electron transport from ATP synthesis. Uncoupling protein 1 (UCP1)[1] was first identified in mammalian brown adipose tissue, where it mediates an uncoupled respiration of fatty acids to generate heat for thermogenesis (2). It makes sense, therefore, that UCP1 is activated by fatty acids, and indeed a proton conductance that is stimulated by fatty acids and inhibited by nucleotides (which act as inhibitors of UCPs) has long been seen as diagnostic of UCP activity (3). A number of homologues of UCP1 exist in animals (2) as well as higher plants (4), fungi and more primitive organisms (5). The function of these homologues is much debated, but their expression in non-thermogenic tissues means that it is unlikely to be related to heat production.

A major advance in our understanding of uncoupling protein function was the discovery that fatty acids do not directly activate UCP. Instead, it was shown that exogenously generated superoxide (in the presence of fatty acids) activates UCPs in both animals (6) and plants (7). To activate UCP, superoxide must be present in the matrix of the mitochondrion, and thus exogenous superoxide must somehow cross the mitochondrial inner membrane (8). Superoxide is an upstream component of the activation pathway of UCP. The end point of this pathway appears to be products of lipid peroxidation such as 4-hydroxy-2-nonenal (HNE) that contain reactive alkenal groups (9). Such compounds are potent activators of animal UCPs. A mechanism has been proposed in which superoxide in the matrix reacts with free Fe^{2+} (which is possibly formed as a result of superoxide attack of iron sulfur-containing proteins such as aconitase) to form the highly reactive hydroxyl radical. This radical initiates carbon-centered lipid radical formation leading to lipid peroxidation that ultimately results in aldehyde degradation products such as HNE that activate UCP (10). Given that mild uncoupling of mitochondria dramatically reduces superoxide formation by the electron transport chain (11), this pathway provides an elegant feedback mechanism by which UCP may act to limit the extent of ROS production in mitochondria.

Although plant UCPs have been shown to be activated by superoxide (7), it is not known whether this superoxide activation proceeds via the HNE pathway that occurs in animals. It is important to establish whether HNE activation of UCP is a feature that is conserved during evolution from plants to animals or whether it evolved since the divergence of the two kingdoms. The former would imply a conservation of function between plant and animal UCP, and thus investigations into the role of UCP in plants may have implications for animal UCP1 homologue function. The latter case (*i.e.* superoxide, but not HNE activation of UCP is conserved between plants and animals) may imply that UCP function in animals is more specifically related to lipid peroxidation rather than ROS *per se*. A relevant suggestion in this regard is that UCP might function as a transporter of oxidized fatty acid molecules (12) although there is not, as yet, any experimental evidence to support this idea.

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[1] The abbreviations used are: UCP, uncoupling protein; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; HNE, 4-hydroxy-*trans*-2-nonenal; $TPMP^+$, methyltriphenylphosphonium; MOPS, 3-morpholinopropanesulfonic acid; ROS, reactive oxygen species; AOX, alternative oxidase; CAT, carboxyatractyloside; ANT, adenine nucleotide translocase; TES, *N*-[Tris(hydroxymethyl)methyl]-2-aminoethane sulfonic acid; TPP, thiamine pyrophosphate.

Here, we investigate whether alkenal products of lipid peroxidation activate UCP in isolated potato tuber mitochondria. By using GTP-inhibitable proton conductance as a measure of UCP activity, or by quantifying proton conductance in mitochondria from transgenic potato plants with increased UCP protein content (7), we conclusively demonstrate that alkenal-containing molecules, such as HNE, activate potato UCP. This result suggests a conservation of the basic mechanism of UCP activation between plants and animals. Further experiments on mitochondria from transgenic plants with increased UCP suggest that under simulated *in vivo* bioenergetic conditions, sufficient activating molecules are generated for UCP to be active. The presence of increased UCP protein content resulted in a modest decrease in the rate of ROS production by isolated mitochondria, and also stimulated the conversion of pyruvate to citrate by the tricarboxylic acid cycle. We suggest that mild uncoupling of the mitochondrial electron transport chain from ATP synthesis as a consequence of UCP activity may not only reduce the rate of mitochondrial ROS production but also may affect tricarboxylic acid cycle flux.

EXPERIMENTAL PROCEDURES

Chemicals—Unless otherwise stated, all chemicals, biochemicals and isotopes were from Sigma-Aldrich. Carboxyatractyloside (CAT) was from Merck Biosciences Ltd. (Nottingham, UK).

Plant Material—Potato tubers (*Solanum tuberosum* L c.v. Desirée) were either purchased from the local supermarket or transgenic tubers were harvested from plants growing in controlled environment glasshouses. Transgenic potato plants overexpressing *StUCP*, lines 18 and 63, (7) were grown by planting sprouted tubers in 150-mm diameter pots containing general purpose compost and sand (2:1). The plants were maintained in a glasshouse at 16–25 °C with a 16-h photoperiod of natural daylight supplemented to give a minimum irradiance of 150 μE m^{-2} s^{-1}. Tubers were harvested after 10 weeks and stored at 22 °C for at least 1 week prior to use. All experiments were done on both transgenic plant lines (lines 18 and 63) but for clarity, only data from line 18 are shown.

Isolation of Mitochondria—Mitochondria were isolated from potato tubers using differential centrifugation and Percoll density gradient centrifugation (7).

Measurement of Proton Conductance—Proton conductance was determined by simultaneous measurement of oxygen consumption and mitochondrial membrane potential using electrodes sensitive to oxygen and the potential-dependent probe, $TPMP^+$ as described previously (13). A reaction chamber of capacity 2 ml was constructed such that the mitochondrial suspension was in contact with both electrodes. Mitochondria (400 μg) were resuspended in 2 ml of assay medium (0.3 M mannitol, 10 mM Hepes-KOH, 1 mM $MgCl_2$, 100 mM KCl, 10 mM KH_2PO_4 (pH 7.0) containing 1 μM oligomycin, and 0.1 μM nigericin. The electrode was calibrated with sequential additions of $TPMP^+$ to a final concentration of 5 μM. Then NADH was added to a concentration of 1 mM to start the reaction. Membrane potential was progressively inhibited by the addition of KCN to a final concentration of between 0.6–20 μM. At the end of each run, 2 μM FCCP was added to dissipate the membrane potential completely, releasing all the $TPMP^+$ into the medium and allowing correction for any small electrode drift. GTP (final concentration, 2 mM), HNE (final concentration 30 μM) (Merck Biosciences Ltd., Nottingham, UK) and *trans*-retinal (final concentration, 5 μM) were added as indicated.

Assay of H_2O_2 Production by Isolated Mitochondria—Hydrogen peroxide was measured using the peroxidase-dependent conversion of Amplex Red (Invitrogen Ltd, Paisley, UK) to the fluorescent compound, resorufin. The assay consisted of the following in a final volume of 200 μl: 50 μM Amplex Red, 10 units superoxide dismutase (VWR International, Lutterworth, UK), 1.2 units of horseradish peroxidase, 0.3 m mannitol, 10 mM Tes-KOH (pH 7.5), 3 mM $MgSO_4$, 10 mM NaCl, 5 mM KH_2PO_4, 0.1% (w/v) bovine serum albumin, 20 mM glucose, 0.3 mM NAD^+, 0.1 mM ADP, 0.1 mM TPP, 0.15 units/ml of hexokinase (Roche Applied Science, Lewes, East Sussex, UK), and mitochondria (30 μg of protein). The background rate of H_2O_2 production was determined by measuring the rate of accumulation of resorufin by fluorescence spectrophotometry (excitation at 563 nm, emission at 587 nm) at a temperature of 25 °C. Respiratory substrates were then added (10 mM pyruvate, 2.1 mM citrate, 1.3 mM succinate, 0.6 mM malate, 0.02 mM fumarate, and 0.02 mM isocitrate) and the rate of hydrogen peroxide production measured. The rate was measured over a time period of 15–30 min after addition of substrates (such that the small amount of ADP added to the incubation medium to initiate ATP synthesis was used up and ADP was produced by the hexokinase-regenerating system).

^{13}C NMR Spectroscopy—Proton-decoupled ^{13}C NMR spectra of low density mitochondrial suspensions were recorded at either 75.46 MHz on a Bruker CXP 300 spectrometer or 150.9 MHz on a Varian Unity Inova 600 spectrometer, in both cases using a 10-mm diameter broadband probe head. The suspension was oxygenated with an air-lift (14), and NMR spectra were recorded in 15-min blocks over a period of 4 h using acquisition conditions similar to those described elsewhere (15). Mitochondria were suspended at a density of 70–100 μg of mitochondrial protein per ml in a buffer containing 0.2 M mannitol, 0.1 mM MOPS, 5 mM $MgCl_2$, 0.1% w/v bovine serum albumin, and 20 mM KH_2PO_4 in 10% D_2O, corrected to pH 7.2 with KOH. The metabolism of 10 mM [3-^{13}C]pyruvate (Aldrich Chemical Company) was observed in the presence of 20 mM glucose, 2.1 mM citrate, 1.3 mM succinate, 0.6 mM malate, 0.3 mM NAD^+, 0.1 mM ADP, 0.1 mM TPP, 0.02 mM fumarate, 0.02 mM isocitrate, and 0.1 units/ml of hexokinase (Roche Applied Science). Glucose and hexokinase were included to regenerate ADP (15) and the tricarboxylic acid cycle acids were added to mimic the cytosolic composition of potato tubers (16).

Western Blotting—Mitochondrial protein was fractionated using SDS-PAGE, transferred to nitrocellulose, and probed with an antibody raised against soybean UCP as described previously (7).

Measurement of Tricarboxylic Acid Cycle Enzymes—the maximal catalytic activities of aconitase, citrate synthase, fumarase, and malate dehydrogenase in isolated mitochondria were determined using NAD(P)H-linked spectrophotometric assays (17).

RESULTS

Potato UCP Is Activated by Products of Lipid Peroxidation—The lipid peroxidation product, HNE, is a potent activator of mammalian UCP (9). It has been proposed that HNE is the end point of an activation pathway that begins with the production of mitochondrial superoxide (10). Superoxide has also been demonstrated to activate UCP activity in plants (7). It is of interest, therefore, to establish whether the mechanism of superoxide activation of UCP is conserved between plants and animals. To this end, we investigated the effect of HNE on UCP-mediated proton conductance in isolated potato mitochondria (Fig. 1*A*). Proton conductance kinetics were determined as membrane potential was titrated with KCN. Oligomycin was added to inhibit ATP synthase activity such that the rate of oxygen consumption was proportional to the rate of proton leak (7). Thus respiration rate gives an indirect measure of proton conductance that is dependent upon the exact H^+/O stoichiometry. To avoid systematic errors as a result of erroneous assumptions about H^+/O stoichiometry, we have not calculated an actual proton conductance rate, but instead present all data as respiration rate. Thus, when proton conductance rate is referred to, it is important to be aware that we are actually presenting the proportional parameter of respiration rate. The kinetic plot of proton conductance as the membrane potential was varied showed that the addition of micromolar concentrations of HNE resulted in a substantial increase in proton conductance rate in comparison to the basal proton leak (Fig. 1*A*). The results are not complicated by the presence of the alternative oxidase (AOX) since AOX is present only at extremely low levels in potato tubers (7). At a membrane potential of 130 mV, respiration rate in the presence of HNE was 268 ± 37 nmol of O min^{-1} (mg of protein)$^{-1}$ and in the absence of HNE, 128 ± 24 ($p < 0.05$, Student's t test). This effect was maximal at a concentration of 30 μM HNE. Preincubation of mitochondria with 2 mM GTP decreased the stimulatory effect of HNE on proton conductance. This suggests that the stimulation of proton conductance by HNE is primarily mediated by activation of UCP. This activation does not require the presence of free fatty acids, either exogenous or endogenous (the results were not

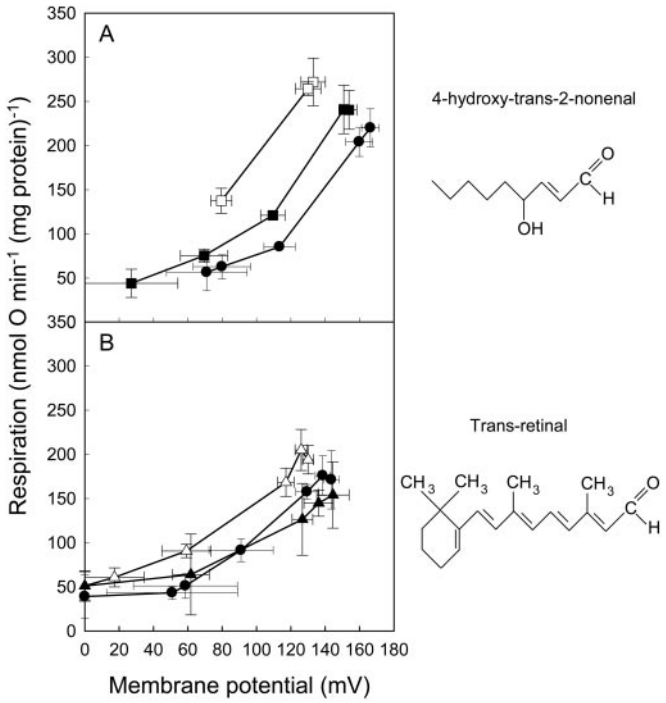

FIG. 1. **HNE and *trans*-retinal induce a GTP-sensitive proton conductance in isolated potato mitochondria.** Potato tuber mitochondria were incubated with 1 mM NADH as a substrate in a medium containing oligomycin to inhibit ATP synthase and nigericin to clamp ΔpH. Under such conditions the rate of respiration is proportional to proton conductance. The kinetics of proton conductance was examined by simultaneous measurements of respiration and membrane potential as the latter was titrated with KCN. Respiration is measured as nmol of atomic oxygen consumed per min per mg of mitochondrial protein. *A*, (●), basal; (□), plus HNE to a final concentration of 30 μM; (■) plus GTP and HNE to final concentrations of 2 mM and 30 μM, respectively. *B*, (●), basal; (△), plus *trans*-retinal to a final concentration of 5 μM; (▲), plus GTP and *trans*-retinal to final concentrations of 2 mM and 5 μM, respectively. All values are the mean ± S.E. of three independent samples.

altered by the addition of BSA to the incubation medium; data not shown). All these assays were done in the presence of 100 μM carboxyatractyloside (CAT), a specific inhibitor of the adenine nucleotide translocase (ANT). This inhibitor was added because the adenine nucleotide translocase can also catalyze a proton conductance and has been shown to be activated by HNE (9). In the absence of carboxyatractyloside, the HNE-stimulated proton conductance of potato mitochondria was not inhibitable by GTP (data not shown). Under these conditions, therefore, it appears that ANT is the dominant protein that contributes to proton conductance, and the effect of UCP is masked. For this reason, carboxyatractyloside was included in all subsequent experiments unless otherwise stated.

In mammalian mitochondria, the structural requirement of the UCP activator molecule is the presence of a reactive 2-alkenal group (9). If this is also the case for plant UCP, then one would expect that other 2-alkenal-containing molecules would also activate plant UCP. *trans*-Retinal is structurally distinct from HNE but also contains a reactive 2-alkenal group (see Fig. 1) and has been shown to activate mammalian UCP (9). The addition of *trans*-retinal to potato mitochondria caused an increase in the proton conductance rate (Fig. 1*B*) although the extent of the increase was less than observed with HNE. At a membrane potential of 130 mV, respiration rate (proton conductance) in the presence of *trans*-retinal was 193 ± 22 nmol of atomic oxygen min^{-1} (mg of protein)$^{-1}$ and in the absence of *trans*-retinal, 135 ± 21 ($p < 0.05$, Student's *t* test). The stimulation of respiration by *trans*-retinal was abolished by preincubation of mitochondria with GTP.

The fact that GTP inhibits the effects of both HNE and *trans*-retinal on proton conductance strongly suggests the involvement of UCP (as GTP inhibits UCP activity). To formally prove that 2-alkenal-containing compounds activate plant UCP, the effect of these compounds on proton conductance of mitochondria from transgenic potato plants overexpressing the potato *UCP* gene *StUCP* was investigated. In confirmation of the observations presented in Fig. 1, both HNE and *trans*-retinal increased the rate of proton conductance above the basal rate in wild-type potato mitochondria (Fig. 2, *A* and *B*) although the increase was smaller than in previous experiments (see Fig. 1). One explanation for this discrepancy may be the source of material. In the experiments shown in Fig. 1, mitochondria were isolated from potatoes purchased from a local supermarket, whereas the wild-type mitochondria used in the experiments shown in Fig. 2 were isolated from tubers harvested from plants grown in a controlled environment glasshouse. In the latter case, tubers were used within 2 weeks of harvesting. In the former, the storage history of the tubers is unknown, but it is likely that they had undergone a considerable period of cold storage. Since cold storage is known to induce UCP protein content in potato tubers (18), it may be that there is an increased UCP content in the supermarket potatoes in comparison to the greenhouse potatoes, and this could be responsible for the greater effect of HNE and *trans*-retinal in mitochondria from the former. This hypothesis was confirmed when the UCP content of mitochondria from the respective potatoes was examined by Western blot (Fig. 3). The intensity of the signal is greater in mitochondria from supermarket potatoes than from glasshouse-grown wild type (quantification of the signal on the Western blot gave the following band intensities: supermarket potatoes, 1390; glasshouse-grown wild type, 995 (arbitrary units)). In contrast to the single band visible in the latter, there appears to be a doublet in the mitochondria from the supermarket tubers. It is not known what the origin of the second band is, but its presence emphasizes the difference between the two sources of tuber.

The level of UCP protein in mitochondria from transgenic tubers overexpressing *StUCP* is much higher than the comparable wild type (band intensities: wild type, 995; transgenic line, 2407(arbitrary units)), confirming previous observations of this transgenic line (7). The stimulatory effect of HNE and *trans*-retinal in mitochondria from these transgenic tubers was markedly increased in comparison to wild type confirming that 2-alkenal-containing compounds activate UCP (compare Fig. 2, *C* and *D* with *A* and *B*). At a membrane potential of 180 mV, respiration rate (proton conductance) in the presence of HNE was 50 ± 7 nmol of atomic oxygen min^{-1} (mg of protein)$^{-1}$ in wild-type mitochondria and 238 ± 36 nmol of atomic oxygen min^{-1} (mg of protein)$^{-1}$ in mitochondria from tubers overexpressing *StUCP* ($p < 0.05$, Student's *t* test). Similarly, in the presence of *trans*-retinal, respiration rate (proton conductance) was 50 ± 14 nmol of atomic oxygen min^{-1} (mg of protein)$^{-1}$ in wild-type mitochondria and 163 ± 39 nmol of atomic oxygen min^{-1} (mg of protein)$^{-1}$ in mitochondria from tubers overexpressing *StUCP* ($p < 0.05$, Student's *t* test).

The stimulatory effect of both HNE and retinal in the transgenic background was inhibited by GTP but for the sake of clarity, the data is not shown. The effect of HNE and *trans*-retinal on proton conductance was similar in a second independent transgenic line overexpressing StUCP (data not shown).

Increased UCP Protein Content Affects Tricarboxylic Acid Cycle Flux in Isolated Mitochondria under Simulated in Vivo Bioenergetic Conditions—It is evident that reactive oxygen species and alkenal products of lipid peroxidation activate both

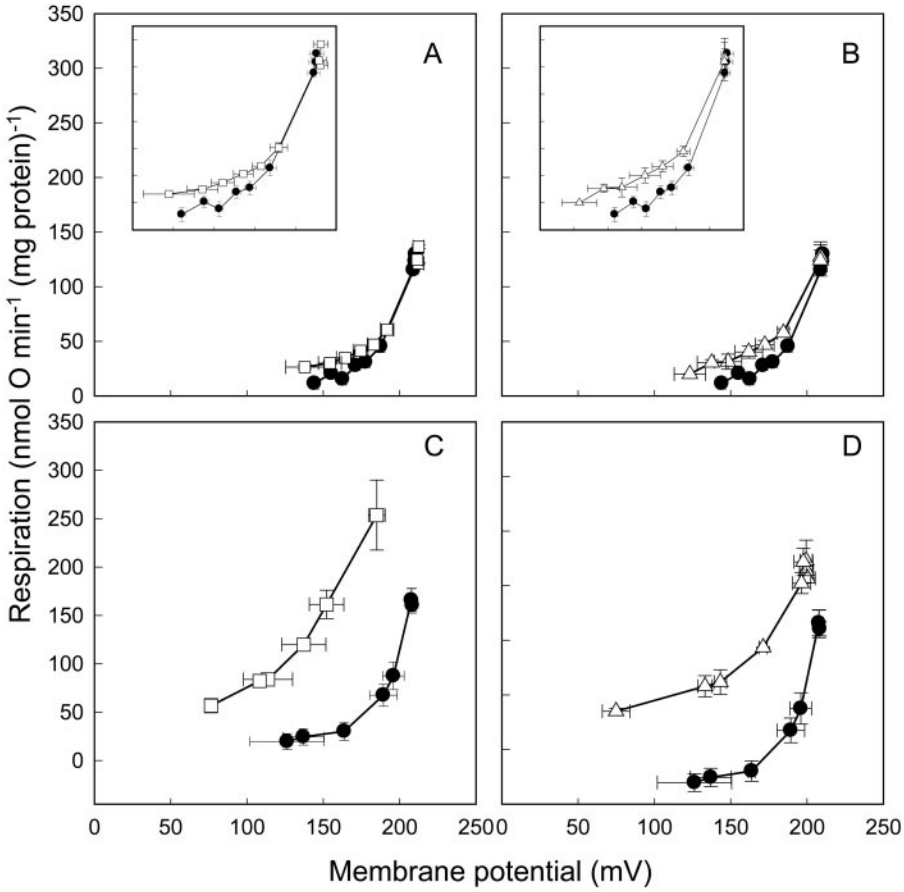

FIG. 2. **Stimulation of proton conductance by HNE and *trans*-retinal is greater in mitochondria from transgenic potato plants with increased StUCP protein content.** Potato tuber mitochondria were incubated with 1 mM NADH as a substrate in a medium containing oligomycin to inhibit ATP synthase and nigericin to clamp ΔpH. Under such conditions the rate of respiration is proportional to proton conductance. The kinetics of proton conductance was examined by simultaneous measurements of respiration and membrane potential as the latter was titrated with KCN. Respiration is measured as nmol of atomic oxygen consumed per min per mg of mitochondrial protein. *A* and *B*, mitochondria from wild-type potato tubers. *C* and *D*, mitochondria from transgenic potato tubers overexpressing *StUCP*. Symbols: (●) basal; (□) plus HNE to a final concentration of 30 μM; (△) plus *trans*-retinal to a final concentration of 5 μM. All values are the mean ± S.E. of three independent samples. The *insets* in *A* and *B* show the same data at an expanded scale for clarity.

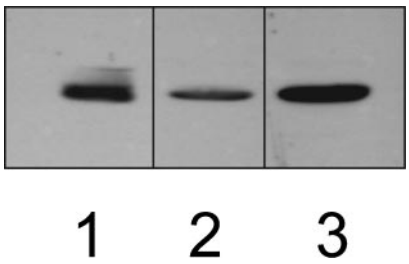

FIG. 3. **Western blot showing UCP protein content of mitochondria used for proton conductance measurements (see legends to Figs. 1 and 2).** *Lane 1*, pooled sample of wild-type mitochondria from which the proton conductance data shown in Fig. 1 were derived. *Lane 2*, pooled sample of wild-type mitochondria from which the proton conductance data shown in Fig. 2, *A* and *B* were derived. *Lane 3*, pooled sample of mitochondria from transgenic potatoes overexpressing *StUCP* (relative to the comparable wild type shown in *lane 2*) from which the proton conductance data shown in Fig. 2, *C* and *D* were derived.

animal and plant UCP when applied exogenously to mitochondria held in an artificial resting bioenergetic state (where ATP synthesis is prevented by absence of ADP and by use of the ATP synthase inhibitor, oligomycin). However, it is not clear whether UCP is activated in mitochondria that are actively synthesizing ATP. To investigate this and to assess the function of UCP in relation to mitochondrial metabolism, we established a system in which the metabolic activity of isolated mitochondria could be monitored in real time by ^{13}C NMR. Isolated mitochondria were incubated in the sample chamber of the NMR spectrometer in a continuously oxygenated buffered solution. [3-^{13}C]Pyruvate was introduced and ^{13}C NMR spectra acquired every 15 min to monitor the metabolism of the labeled pyruvate. To spark the tricarboxylic acid cycle, unlabeled tricarboxylic acid cycle acids were added to the incubation medium at concentrations that are observed in the cytosol of potato tubers (16). An ADP-regenerating system consisting of 20 mM glucose and 0.1 units/ml hexokinase was also included. Nigericin, oligomycin, and carboxyatractyloside were not added. The amount of hexokinase required for maximal regeneration of ADP was established by titrating hexokinase amount and measuring respiration rate (data not shown). This system simulates *in vivo* conditions in which mitochondria are respiring pyruvate and in which there is a demand for ATP. The oxygen consumption rate was constant during the period of the experiment (Fig. 4*A*). ^{13}C-Label from pyruvate accumulated in several tricarboxylic acid cycle acids (Fig. 4*B*). It is important to realize that because of the low volume of the mitochondrial matrix in the sample and its highly proteinaceous nature, it is not possible to detect ^{13}C NMR signals from molecules from within the mitochondria. Hence the NMR peaks represent compounds that have been exported from the mitochondrion into the incubation medium. Lysing the mitochondria at the end of the labeling period did not increase the signal from ^{13}C-labeled tricarboxylic acid cycle acids, indicating that the majority of the labeled acids exchange rapidly with the external medium rather than being retained in the mitochondria (data not shown). This is relevant because it means that a perceived increase in the rate of labeling of tricarboxylic acid cycle acids reflects increased tricarboxylic acid cycle flux rather than a change in the rate of export of labeled compounds from the mitochondrial matrix into the medium.

The relationship between the extent to which mitochondria are coupled (that is the extent to which electron transport flux is coupled to the ATP synthase reaction) and flux through the tricarboxylic acid cycle has not previously been empirically examined. It is generally assumed that electron transport flux imposes a limit on the tricarboxylic acid cycle flux due to the

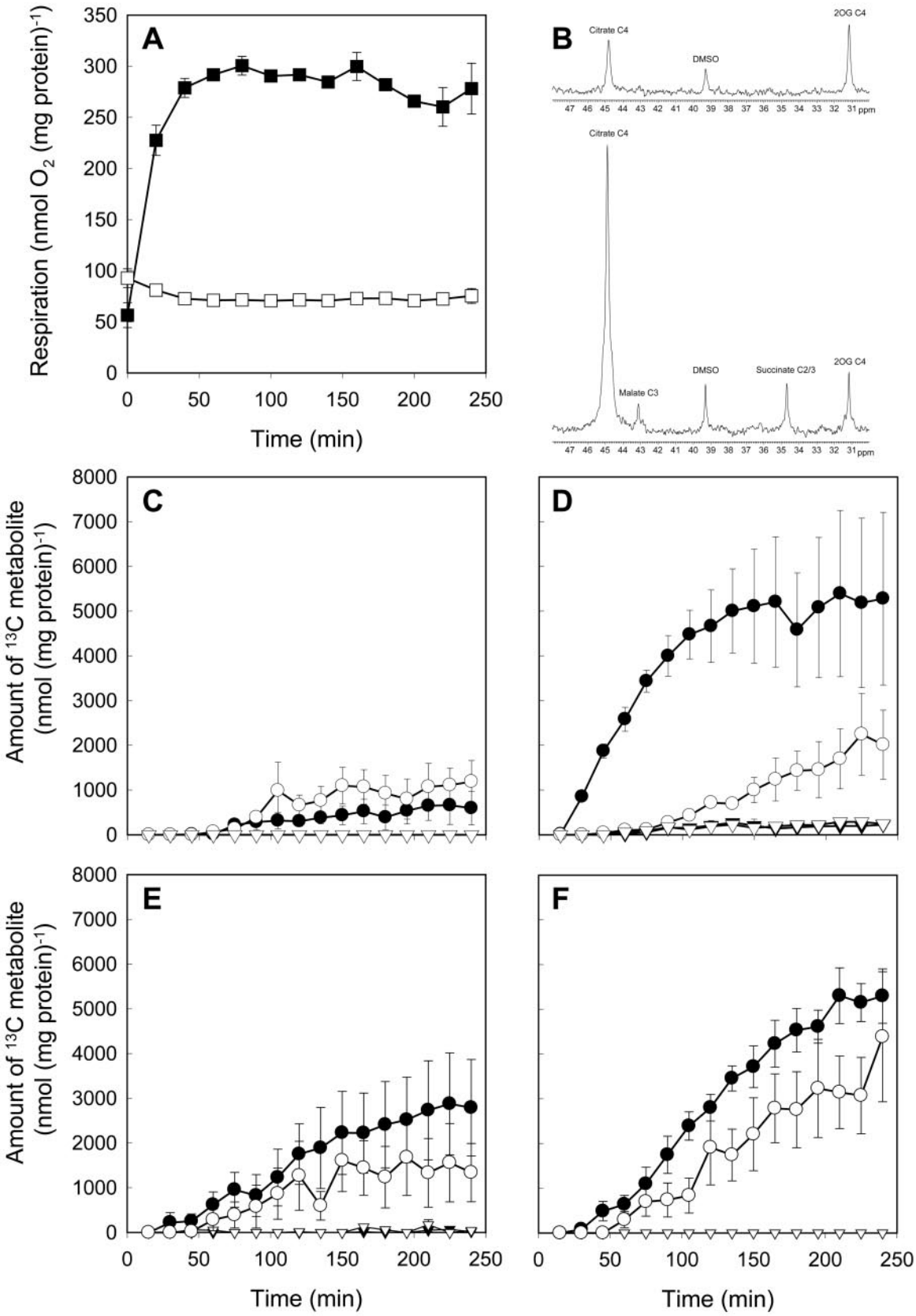

FIG. 4. **Uncoupling increases the flux from pyruvate to citrate in isolated mitochondria.** Mitochondria were incubated in a buffered medium containing pyruvate and tricarboxylic acid cycle organic acids to mimic cytosolic conditions as well as an ATP sink. *A*, the rate of oxygen consumption (nmol of molecular oxygen min^{-1} mg of mitochondrial $protein^{-1}$) in the presence of (□) 0 μM and (■) 4 μM CCCP. All values are the mean ± S.E. of four independent samples. *B*, ^{13}C NMR spectra of isolated mitochondria respiring 10 mM [3-^{13}C]pyruvate 0 μM (*upper spectrum*) and 4 μM CCCP (*lower spectrum*). Each spectrum is the sum of a series of spectra recorded over 4 h. *C–F*, the production of [4-^{13}C]citrate (●), 2-oxo-[4-^{13}C]glutarate (○), [2/3-^{13}C]succinate (▽), and [3-^{13}C]malate (▼) by mitochondria in the absence of CCCP (*C*), mitochondria in the presence of 4 μM CCCP (*D*), UCP wild-type mitochondria (*E*), and UCP line 18 mitochondria (*F*). Measurements are based on ^{13}C NMR signal peak intensities and values are the mean ± S.E. of three independent samples.

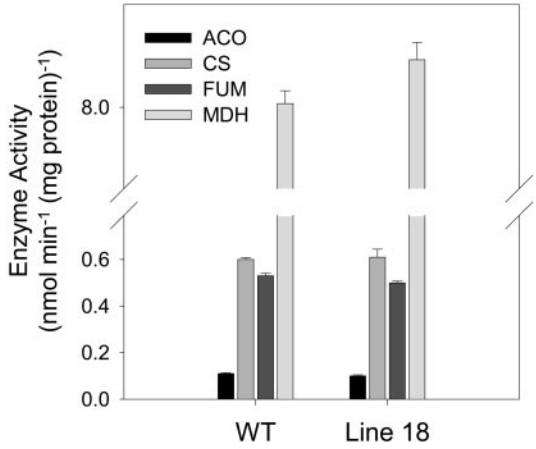

FIG. 5. **Maximal catalytic activities of tricarboxylic acid cycle enzymes in mitochondria from wild-type and transgenic potato plants overexpressing StUCP.** Legend abbreviations: *ACO*, aconitase; *CS*, citrate synthase; *FUM*, fumarase; *MDH*, malate dehydrogenase). All values are the mean ± S.E. of three independent samples.

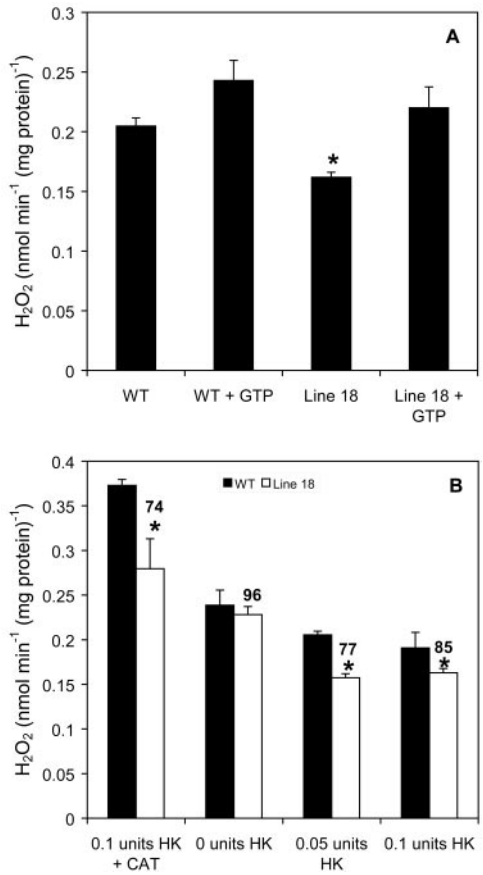

FIG. 6. **Rate of superoxide production in mitochondria from wild-type and transgenic plants overexpressing StUCP.** Superoxide was detected in isolated mitochondria following its dismutation to hydrogen peroxide by both endogenous and exogenous superoxide dismutase. Hydrogen peroxide was detected using the peroxidase conversion of Amplex Red to the fluorescent resorufin. Mitochondria were incubated in a medium containing pyruvate and tricarboxylic acid cycle acids at *in vivo* cytosolic concentrations as well as an ATP sink (for more details, see "Experimental Procedures"). *A*, hydrogen peroxide production by mitochondria from wild-type and transgenic potato plants overexpressing *StUCP* in the presence and absence of GTP (final concentration of 2 mM as indicated). 0.1 units/ml of hexokinase were added as an ATP sink and ADP-regenerating system. *B*, the effect of varying the strength of the ATP sink on hydrogen peroxide production in wild-type and transgenic mitochondria. The incubation medium contained different amounts of hexokinase (*HK*) as indicated. CAT was also added where indicated. *Closed bars* are wild-type, and *open bars* are line 18. The numbers *above* the open bars indicate the value as a percentage of wild-type. * indicates significantly different to wild-type (under comparable incubation conditions) ($p < 0.05$, Student's t test). All values are the mean ± S.E. of three independent samples.

rate of regeneration of NAD^+ by complex I. However, in plant mitochondria, there are a number of rotenone-insensitive NADH and NADPH dehydrogenases whose activity is not linked to proton translocation (19) as well as an alternative non-phosphorylating terminal oxidase (20) that could combine to form a NAD-regenerating electron transport chain that is uncoupled from ATP synthase acitivity (21). To establish whether uncoupling mitochondria affects respiration and tricarboxylic acid cycle metabolism, we added the classic uncoupler CCCP (Fig. 4, *A–D*). This led to a 3-fold increase in the rate of respiration (Fig. 4*A*) and had a marked effect on the rate of accumulation of ^{13}C label in tricarboxylic acid cycle intermediates, with appreciably more label accumulating in all detected tricarboxylic acid cycle acids, with a particularly large increase in citrate (see Fig. 4, *C* and *D*). Thus, it can be concluded that uncoupling mitochondria leads to an increase in tricarboxylic acid cycle flux, particularly in the flux from pyruvate to citrate.

This effect of uncoupling on tricarboxylic acid cycle flux provides a tool to test whether UCP is active under these simulated *in vivo* bioenergetic conditions. Tricarboxylic acid cycle metabolism was compared in mitochondria from wild-type potato tubers and from those overexpressing the *StUCP* gene (Fig. 4, *E* and *F*). If UCP is active in these mitochondria, then one would predict that overexpression of StUCP would reduce the extent to which mitochondria are coupled and this would lead to an increased tricarboxylic acid cycle flux. This indeed appears to be the case, with appreciably more label accumulating in both citrate and 2-oxoglutarate in the mitochondria from the transgenic line. The rate of accumulation of labeled citrate was estimated from the slope of the curve during the phase of linear accumulation between 45 and 150 min. There was a statistically significant increase in the transgenic line overexpressing UCP (Student's t test, $p < 0.1$): wild type = 18 ± 7 nmol min^{-1} mg $protein^{-1}$, transgenic = 34 ± 4 nmol min^{-1} mg $protein^{-1}$. This suggests that UCP is active in mitochondria under simulated *in vivo* bioenergetic conditions. Similar results were obtained with a second independent transgenic line. To check that these changes in tricarboxylic acid cycle metabolism were related to the coupling state of the mitochondria and not due to long term changes in tricarboxylic acid cycle enzyme activities because of expression of the UCP transgene, we assayed the activity of selected tricarboxylic acid cycle enzymes in mitochondria from wild-type and transgenic tubers (Fig. 5). There were no significant differences in the maximal catalytic acitivities of aconitase, citrate synthase, fumarase, or malate dehydrogenase per mg of mitochondrial protein.

Increased UCP Protein Content Reduces the Production of Superoxide by Mitochondria under Simulated in Vivo Bioenergetic Conditions—One of the proposed functions of UCP activity is to minimize the production of reactive oxygen species by the mitochondrial electron transport chain (7, 22). Previously it has been shown that addition of linoleic acid to isolated potato mitochondria respiring succinate reduced the production of hydrogen peroxide (23). However, the possibility that the effect of linoleic acid in these experiments was related to proteins other than UCP cannot be ruled out, particularly as the linoleic effect was not shown to be GTP/ATP inhibitable. To investigate the linkage between UCP amount and reactive oxygen species production in isolated mitochondria directly, we measured the rate of H_2O_2 production in mitochondria isolated from wild-type and transgenic tubers overexpressing StUCP (Fig. 6).

Hydrogen peroxide is produced from superoxide by the action of superoxide dismutase (SOD). The rate of production of hydrogen peroxide is therefore an indirect measure of mitochondrial superoxide production. Addition of exogenous superoxide dismutase did not increase the measured rate of hydrogen peroxide production (data not shown) indicating that the superoxide is formed exclusively in the matrix. Mitochondria were incubated in the same conditions as described for the ^{13}C-labeling experiments to simulate an *in vivo* bioenergetic state. Under these conditions there was a modest, but significant (Student's *t* test, $p < 0.05$) decrease in the rate of hydrogen peroxide production in the transgenic line (Fig. 6*A*). Furthermore, preincubation of mitochondria with GTP to inhibit UCP activity abolished the difference in rate of hydrogen peroxide production (Fig. 6*A*). Similar results were obtained with a second independent transgenic line.

These results demonstrate that UCP is active under simulated *in vivo* conditions which implies that sufficient superoxide/HNE are present in mitochondria in this bioenergetic state to at least partially activate UCP. Since superoxide production is dependent upon membrane potential (11) one might predict that UCP would become more active as membrane potential increases. To test this, we varied the strength of the ATP sink (and ADP-regenerating system) by reducing the amount of hexokinase in the incubation medium. This will reduce the regeneration of ADP thereby decreasing the activity of the ATP synthase complex and causing an increase in membrane potential. Based on a titration of hexokinase amount against respiration (data not shown) the following amounts of hexokinase were used: (no hexokinase (equivalent to state IV), 0.05 units/ml hexokinase (corresponding to half saturating amount), and 0.1 units/ml hexokinase (corresponding to a saturating amount of hexokinase). The rate of hydrogen peroxide production by isolated mitochondria incubated with pyruvate and cytosolic concentrations of tricarboxylic cycle acids was measured (Fig. 6*B*). As one would expect, there was an increase in the rate of hydrogen peroxide production as the amount of added hexokinase was decreased. A comparison of the hydrogen peroxide production rate between mitochondria from wild-type plants and those overexpressing UCP (line 18) gives an indication of the activation state of UCP. As before (Fig. 6*A*), at 0.1 units/ml hexokinase there was a significant decrease in the rate of hydrogen peroxide production in mitochondria from line 18. When the hexokinase concentration was reduced to 0.05 units/ml the decrease is greater still (77% of wild type at 0.05 units/ml of hexokinase compared with 85% at 0.1 units/ml hexokinase) suggesting that UCP is more active. However, this trend does not continue through to the condition in which hexokinase was omitted (equivalent to state IV) in which there was now no difference in hydrogen peroxide production between wild type and line 18. One possible explanation for this is that the proton-conducting activity of ANT is active under this condition and masks the effect of UCP. This possibility was confirmed by the observation that addition of carboxyatractyloside to inhibit ANT restored the difference in hydrogen peroxide production between wild type and line 18.

DISCUSSION

The Mechanism of Activation of UCP Is Conserved between Plants and Animals—Addition of micromolar concentrations of HNE to potato tuber mitochondria caused an increased proton conductance that was inhibitable by GTP (Fig. 1). The extent of HNE-induced proton conductance was enhanced in mitochondria from transgenic plants overexpressing StUCP, confirming that this effect is related to UCP (Fig. 2). A similar activation of UCP is apparent after incubation with *trans*-retinal. The activation of potato UCP by HNE and *trans*-retinal suggest, that as is the case with animal UCP (9), the terminal alkenal group that is common between HNE and *trans*-retinal is the structural requirement for activation. It seems very likely, therefore, that mitochondrial ROS activate plant UCP by initiating lipid peroxidation, with the alkenal end products of this process being the activating molecules. However, the data presented here do not discount the possibility that other activated molecules such as carbon-centered lipid radicals may activate plant UCP directly. The conservation of the mechanism of activation of UCP between plants and animals may imply that the role of UCP in plants and UCP1 homologues in animals is similar.

Potato UCP Is Active in Isolated Mitochondria under Simulated in Vivo Bioenergetic Conditions—Identification of HNE as an activator of plant UCP gives an indication as to the signaling pathway that governs UCP activity, but does not establish the conditions under which UCP is active *in vivo*. It has been demonstrated that rat mitochondria can produce sufficient endogenous superoxide from complex I to activate UCP but the bioenergetic conditions required for this ROS production do not relate to those likely to occur *in vivo* (24). We assessed activation of UCP in mitochondria held in a bioenergetic state closer to that which is likely to occur *in vivo*. Specifically, mitochondria were respiring pyruvate such that the points of entry of electrons into the electron transport chain were from endogenous NADH and succinate produced by the tricarboxylic acid cycle. In addition, ATP synthase was active and an exogenous ATP sink was generated by addition of hexokinase and glucose. Finally, exogenous tricarboxylic acid cycle acids were supplied at concentrations known to occur in potato cytosol such that exchange of organic acids between matrix and the medium mimics that of matrix and cytosol.

Under these conditions, we demonstrate that uncoupling of electron transport from ATP synthesis had a significant effect on tricarboxylic acid cycle flux. Addition of an uncoupler (CCCP) caused a marked increase in the rate of oxygen consumption and led to an appreciable increase in the rate of metabolism of pyruvate to tricarboxylic acid cycle intermediates (Fig. 4). This suggests that even though ATP synthesis is active, the ATP synthase reaction is not operating at maximal capacity (State III) as the increase in respiration following the addition of uncoupler is much larger under these *in vivo* conditions than when added to mitochondria at state III (data not shown). Presumably, the rate of regeneration of ADP by the exogenous hexokinase reaction is effectively limiting the activity of the ATP synthase complex in this system. It appears that in such circumstances, the activity of ATP synthase imposes a restriction on the rate of regeneration of matrix NAD^+ by complex I and this in turn limits tricarboxylic acid cycle flux. Uncoupling of mitochondria relieves the thermodynamic constraint on electron transport, allows a higher activity of NAD^+ recycling and results in a higher tricarboxylic acid cycle flux. This experimental set up provides a system to test the extent to which UCP is active under bioenergetic conditions that are more like those that may occur *in vivo*. If UCP is active under such conditions, then there should be a relationship between UCP protein content and tricarboxylic acid cycle flux. Comparison of mitochondria from wild type and transgenic plants overexpressing UCP confirmed this to be the case, with a significant increase in the rate of metabolism of pyruvate to citrate in the mitochondria with increased UCP protein content (Fig. 4). Tricarboxylic acid cycle enzymes were unchanged in the transgenic plants (Fig. 5) and thus the increased tricarboxylic acid cycle flux appears to be directly related to UCP protein content.

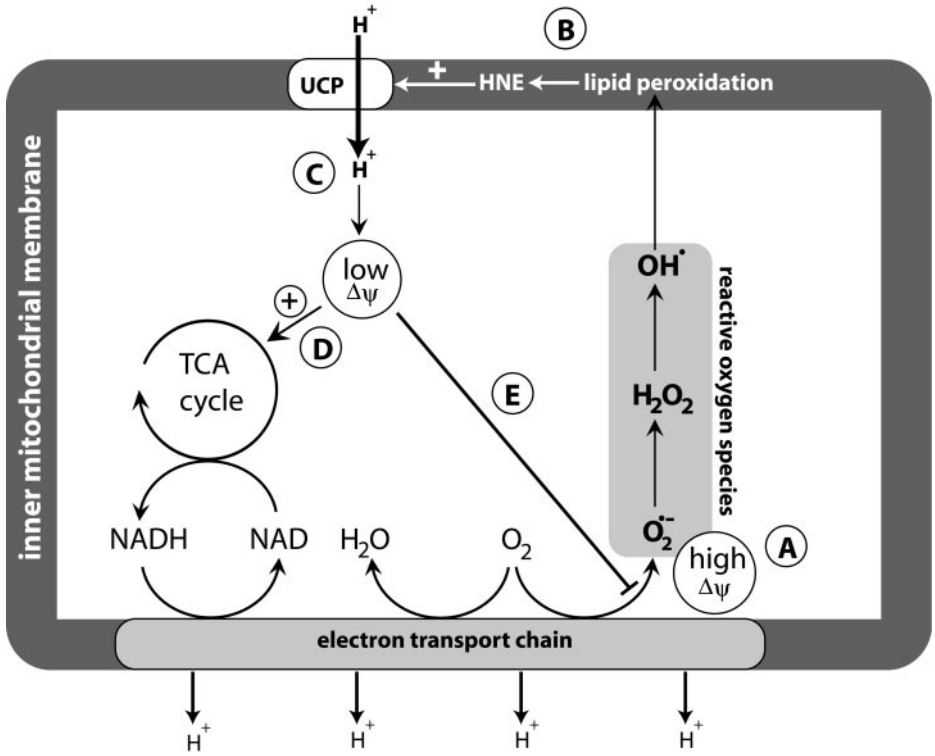

FIG. 7. **Scheme of the interactions between mitochondrial membrane potential, ROS, and HNE production, UCP activity, and tricarboxylic acid cycle flux.** High mitochondrial membrane potential ($\Delta\psi$) leads to increased ROS production (*A*). Hydroxyl radicals initiate peroxidation of inner membrane lipids leading to production of HNE and other reactive alkenals (*B*). HNE activates UCP, which translocates protons from the intermembrane space into the matrix leading to a reduction in $\Delta\psi$ (*C*). Reduced $\Delta\psi$ facilitates higher tricarboxylic acid cycle flux (*D*) and reduces ROS production (*E*). Thus ROS act as a signal that activates a mechanism involving UCP to reduce ROS production. The activation of UCP by HNE and the consequences for $\Delta\psi$, tricarboxylic acid cycle flux, and ROS production are demonstrated in this study. The pathway leading from superoxide to HNE is inferred from our previous work, which demonstrates activation of potato UCP by superoxide (7) and by studies of the effect of a spin trap that reacts with carbon-centered radicals on proton conductance in animal mitochondria (10).

Further evidence that UCP is active under these conditions was provided by comparison of the rate of superoxide production in mitochondria from wild-type and transgenic plants. We show that increased UCP protein content leads to a modest, but significant decrease in the rate of superoxide production under simulated *in vivo* bioenergetic conditions (Fig. 6*A*). The dependence of UCP activation on the bioenergetic state of the mitochondria was investigated by measuring the rate of hydrogen peroxide production as the strength of the ATP sink was reduced (by reducing the amount of hexokinase in the incubation medium) (Fig. 6*B*). A rather complex picture was obtained in which the proton conducting activity of ANT is also apparent. As hexokinase is reduced from a saturating (0.1 units/ml) to a half-saturating amount (0.05 units/ml), the activation state of UCP increases (the amount of hydrogen peroxide produced in mitochondria from transgenic tubers overexpressing UCP decreases relative to wild type). However, when no hexokinase is included in the incubation medium (equivalent to state IV), this trend is reversed and there is no apparent difference in the rate of hydrogen peroxide production between transgenic and wild type. This result is unexpected because at state IV one would expect the amount of superoxide production by the electron transport chain to be maximal and therefore the activation of UCP to be maximal and the difference between wild type and transgenic to be at its greatest. One possible explanation for this result is that under state IV conditions, ANT contributes a significant proton leak that masks the effect of UCP (see "Results": Potato UCP is activated by products of lipid peroxidation). We therefore created an equivalent state IV condition by incubating with hexokinase but inhibiting ANT with carboxyatractyloside. This increased the amount of hydrogen peroxide in comparison the condition in which hexokinase is absent and importantly, the difference between wild type and the transgenic line overexpressing UCP was at its greatest. These results suggest that the proton conductance activity of ANT is particularly prevalent at state IV but is less apparent when mitochondrial ATP synthesis is occuring. It is possible that when ANT is catalyzing adenine nucleotide exchange its proton conductance activity is inhibited, possibly because protons and adenine nucleotides compete for the same binding site.

What Is the Function of UCP in Vivo?—The only UCP for which there is a definitive answer to this question is mammalian UCP1 which undoubtedly functions to generate heat for thermogenesis. For mammalian UCP1 homologues and for plant UCPs, the picture is less clear. Uncoupling mitochondrial electron transport from ATP synthesis has a number of outcomes including reduction of the rate of ROS formation as well as regulation of tricarboxylic acid cycle flux. Since these outcomes are inextricably linked to mitochondrial coupling, it is difficult to separate them and hence discern the function of UCP in a physiological context. However, since ROS appear to act as a feedback signal that regulates UCP activity (Fig. 7), a role for UCP in regulation of mitochondrial ROS production seems likely. Undoubtedly, inhibition of UCP acitivity or disruption of UCP gene expression causes an increase in ROS production (24, 25). However, this does not prove that the role of UCP *in vivo* is to limit ROS production since complete inhibition of activity or absence of UCP protein is an extreme situation. In this article we show that overexpression of UCP results in only a modest reduction of mitochondrial superoxide. In addition, there was no change in the activity of mitochondrial aconitase activity in transgenic plants overexpressing UCP. Since this enzyme is irreversibly inhibited by mitochondrial ROS (26), this suggests that the basal level of superoxide production is not sufficient for the aconitase protein to be significantly inhibited. In the context of plants, a number of other observations are relevant. First, UCP1 is one of the most abundant inner mitochondrial membrane proteins in Arabidopsis mitochondria (27). Second, in a number of different plant species, UCP gene expression or protein abundance is increased during a variety of stresses (4, 18, 28, 29). Taken together with the results presented in this article, the available evidence supports the view that the capacity of UCP is sufficient to prevent oxidative damage under optimal conditions, but additional UCP protein may be required to minimize oxidative damage under stress conditions.

Our data also highlight a potential role for UCP that is perhaps less often considered: that of facilitating a high tricarboxylic acid cycle flux. In a conventional respiratory pathway, carbon entering the tricarboxylic acid cycle is completely oxidized to CO_2 and the electrons from the resulting reductant (NADH) enter the mitochondrial electron transport chain for ATP synthesis. This couples tricarboxylic acid cycle flux to electron transport (the latter regenerating NAD^+ that is required for continued tricarboxylic acid cycle flux). Electron transport, in turn, is coupled to ATP synthesis and thus tricarboxylic acid cycle flux is also effectively coupled to ATP synthesis. This tight coupling of tricarboxylic acid cycle flux to ATP synthesis makes sense when the sole function of the tricarboxylic acid cycle is catabolic respiration. However, the tricarboxylic acid cycle also has an anabolic role, providing intermediates to support the biosynthesis of a number of molecules including amino acids and terpenoids. Under such circumstances the flux demand on the tricarboxylic acid cycle is greater than required for respiration alone. Therefore, the strict coupling between tricarboxylic acid cycle and ATP synthesis must be moderated. Dissipation of the proton gradient by UCP is one means to achieve this. We show that in bioenergetic conditions in which tricarboxylic acid cycle flux is limited by the extent of mitochondrial coupling, that increased UCP protein content leads to a significant increase in tricarboxylic acid cycle flux.

This role for UCP may be particularly important in autotrophic organisms such as plants, in which the biosynthetic demand on the tricarboxylic acid cycle is great (30). This metabolic role for UCP is also apparent during the re-wiring of metabolism that occurs in plant tissues subject to carbohydrate deprivation (UCP transcript abundance is significantly increased under such conditions) (31). The importance of partial uncoupling of plant mitochondria for metabolic purposes is apparent in the presence of a number of non-phosphorylating by-passes of the electron transport chain that are not present in animals. These proteins (rotenone-insensitive NAD(P) dehydrogenases) and AOX undergo electron transfer redox chemistry without concomitant proton translocation and their activity is not therefore coupled to ATP synthase activity (19, 20). Given the different patterns of expression and regulation of these different routes of mitochondrial uncoupling it is unlikely that these different proteins are merely redundant in function but rather fulfill specific roles in specific cell types and under specific conditions.

The work presented in this article suggests that UCP has a key role in modulating the interrelationships between mitochondrial membrane potential, ROS production and tricarboxylic acid cycle flux. We have brought together the findings of this work with those of previous studies (7, 10) to produce a model that summarizes the role of UCP in modulating the bioenergetic balance within the mitochondrion (Fig. 7). Briefly, a high membrane potential leads to increased ROS production and ultimately to HNE production and UCP activation. Increased proton conductance by UCP reduces the membrane potential and this not only has the effect of reducing ROS production, but may also alleviate thermodynamic restrictions on tricarboxylic acid cycle flux.

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Metabolism and Bioenergetics:

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