Superoxide Stimulates a Proton Leak in Potato Mitochondria That Is Related to the Activity of Uncoupling Protein*

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The ability of plant mitochondrial uncoupling proteins to catalyze a significant proton conductance in situ is controversial. We have re-examined conditions that lead to uncoupling of mitochondria isolated from the tubers of potato (Solanum tuberosum). Specifically, we have investigated the effect of superoxide. In the absence of superoxide, linoleic acid stimulated a proton leak in mitochondria respiring NADH that was insensitive to GTP. However, when exogenous superoxide was generated by the addition of xanthine and xanthine oxidase, there was an additional linoleic acid-stimulated proton leak that was specifically inhibited by GTP. Under these conditions of assay (NADH as a respiratory substrate, in the presence of linoleic acid and xanthine/xanthine oxidase) there was a higher rate of proton conductance in mitochondria from transgenic potato tubers overexpressing the StUCP gene than those from wild type. The increased proton leak in the transgenic mitochondria was completely abolished by the addition of GTP. This suggests that superoxide and linoleic acid stimulate a proton leak in potato mitochondria that is related to the activity of uncoupling protein. Furthermore, it demonstrates that changes in the amount of StUCP can alter the rate of proton conductance of potato mitochondria.

The flux through the tricarboxylic acid cycle and the flow of electrons through the mitochondrial respiratory chain are coupled to the synthesis of ATP via the proton gradient across the inner mitochondrial membrane. The activity of the F0F1 ATP synthase complex returns protons to the matrix allowing continued electron transport (with concomitant proton translocation into the intermembrane space) and recycling of NAD as a substrate for the tricarboxylic acid cycle. This strict coupling of tricarboxylic acid cycle, electron transport, and ATP synthesis matches respiratory activity to ATP demand. However, there are circumstances in which maintaining a coupled state is not desirable. Plants are autotrophic organisms and the tricarboxylic acid cycle is a source of carbon skeletons for a number of biosynthetic pathways (1, 2). Under these conditions, a higher tricarboxylic acid cycle flux is required than is the case for ATP synthesis alone, and the tricarboxylic acid cycle must be partially uncoupled from electron transport. Uncoupling may also be beneficial in reducing the production of reactive oxygen species by the respiratory chain and thereby avoiding oxidative stress (3). The respiratory chain of plant mitochondria therefore contains a number of non-phosphorylating bypasses such as the alternative oxidase and NADP(H) dehydrogenases (4, 5). These electron carriers do not translocate protons and are not dependent upon the dissipation of the proton gradient by ATP synthase.

An alternative means of attaining a regulated uncoupling of plant mitochondria is via the activity of the mitochondrial uncoupling protein (UCP).1 UCP was first discovered in the brown adipose tissue of mammals where it functions to catalyze an uncoupled respiration of fatty acids to generate heat for thermogenesis (6). Consistent with this role, UCP1 is activated by anionic fatty acids, which are thought to directly participate in its catalytic function (7–9). Since then, a number of UCP1 homologues have been discovered in mammals (UCP 2–5), which were initially also assumed to catalyze mitochondrial proton leak (although not necessarily for the generation of heat energy). However, there is still considerable controversy as to whether the UCP1 homologues actually catalyze a proton leak in vivo and the function of these proteins remains to be established (10).

In plants, an activity reminiscent of UCP1 has also been identified (11). This activity was characterized by a reduction in membrane potential of isolated potato mitochondria that was stimulated by anionic fatty acids and inhibited by nucleotides, characteristics that distinguish uncoupling protein from other anion carrier protein-mediated proton leak. Two years later the first plant UCP gene was cloned (StUCP from potato) (12), and since then UCP genes have been identified from Arabidopsis (13, 14), skunk cabbage (15), wheat (16), and rice (17). UCP-like activity has been observed in isolated mitochondria from a number of plant species and the potato UCP activity has been purified and its proton transport properties recovered by reconstitution into liposomes (18). Furthermore, reconstitution of the AtUCP1 gene product into liposomes has provided the first link between a plant UCP gene and proton transport activity (19). However, a recent study has shown that

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1 The abbreviations used are: UCP, uncoupling protein; Tes, 2,3-bis[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid; BSA, bovine serum albumin; AOX, alternative oxidase; WT, wild type; TPMP, triphenylmethylphosphonium.
when care is taken to avoid artifacts caused by extraneous effects of nucleotides on other electron transport chain components, the fatty acid-stimulated proton leak of potato mitochondria is not inhibited by nucleotides (20). This casts considerable doubt as to whether UCP contributes in any significant way to proton leak in plant mitochondria.

In this paper we sought to undertake a careful re-examination of the conditions that lead to proton leak in potato mitochondria and to establish under which conditions, if any, UCP contributes to this proton leak. We confirm previous observations that fatty acids stimulate a proton leak in potato mitochondria respiring NADH and that this leak is not inhibited by nucleotides. However, when exogenous superoxide is generated (by the addition of xanthine and xanthine oxidase) an additional proton leak is observed that is sensitive to GTP. This suggests that, as is the case in animals, plant UCP requires the presence of superoxide for full activity (21, 22). We provide further evidence that this fatty acid-dependent, superoxide-stimulated, and nucleotide-sensitive proton leak is related to UCP activity by studying proton leak in mitochondria isolated from transgenic potato plants overexpressing the potato StUCP gene.

Experimental Procedures

Chemicals—Unless otherwise indicated all chemicals were from Sigma, Poole, UK).

Growth of Potato Plants—Potato (Solanum tuberosum L. c.v. Desiree) were grown by planting sprouted tubers in 150-mm diameter pots containing general purpose compost. 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 4 °C for at least 1 week prior to use.

Production of Transgenic Plants Overexpressing the StUCP cDNA—The full-length StUCP cDNA (12) was cloned into the binary plant expression vector pBinAR (23) between the 35S cauliflower mosaic virus promoter (24) and the polyadenylation signal of the T-DNA octopine synthase gene (25) using standard techniques. The resulting construct was introduced into Agrobacterium tumefaciens and used to transform Solanum tuberosum L. c.v. Desiree as described previously (26).

Northern and Western Blot Analyses—Total RNA was extracted from leaves and analyzed by Northern blot using radiolabeled StUCP as a probe as described previously (12). UCP protein content in isolated mitochondrial samples was assessed by Western blot analysis using an antibody raised against soybean UCP as described in Ref. 27.

Isolation of Potato Tuber Mitochondria—All procedures were done at 4 °C. Approximately 100 g of tuber material was homogenized into 100 ml of extraction medium (0.3 M mannitol, 50 mM Tes-NaOH (pH 7.5), 0.5% (w/v) BSA, 0.5% (w/v) polyvinylpyrrolidone-40, 2 mM EGTA, and 20 mM cysteine) containing 50 μM carbonyl cyanide m-chlorophenylhydrazone to allow the generation of superoxide upon addition of xanthine oxidase induced an additional proton conductance (compare Fig. 1b with 1a). Furthermore, the addition of GTP reduced this rate of proton conductance back to a level similar to that observed in the presence of linoleic acid alone (Fig. 1a, 1b, and 1c). The superoxide effect was not seen in the absence of linoleic acid (data not shown).
antibody raised against soybean UCP (27) (Fig. 2b). To quantitate the increase in UCP protein, we established the linearity of response of the UCP antibody to increasing amounts of mitochondrial protein (data not shown) and loaded appropriate amounts of protein such that the signal for each line was within the linear range (Fig. 2c). Band intensity was determined using the Multianalyst software package (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Band intensity was expressed per milligram of total mitochondrial protein to give a measure of UCP content. We estimated that line 18 contains 13 times as much UCP as WT and line 63, twice as much. The mitochondrial samples used for this analysis were derived from a pool of three independent mitochondrial isolations of each line and were the same mitochondrial samples used for proton leak assays.

We investigated the effect of this increased UCP content on proton conductance (Fig. 3). In the presence of linoleic acid alone the proton leak of mitochondria from the two transgenic lines was indistinguishable from wild type (Fig. 3, a and d). However, when xanthine/xanthine oxidase was added (to generate superoxide) in addition to linoleic acid there was an increased rate of proton conductance in mitochondria from the transgenic lines (Fig. 3, b and e). This increased rate of proton conductance was specifically dependent on the presence of superoxide as the addition of superoxide dismutase returned proton leak to wild type levels (Fig. 3b, inset). Furthermore, the superoxide-stimulated proton conductance was completely abolished by the addition of GTP (Fig. 3c and f). We calculated the rate of proton conductance from the curves shown in Fig. 3 by assuming an H+/H1001/O ratio of 6 for oxidation of external NADH. The proton conductance was calculated at a membrane potential of 130 mV. In comparison to wild type there was a statistically significant increase in proton conductance in the transgenic lines (t test; p < 0.05) of 3.0-fold in line 18 and 2.3-fold in line 63 (Table I).

**DISCUSSION**

Plant genomes contain homologues of mammalian UCPs and these gene products demonstrate UCP-like activity when reconstituted into liposomes or overexpressed in heterologous organisms such as yeast (18–20). This activity is characterized as a fatty acid-stimulated proton conductance that is inhibited by nucleotides such as ATP and GTP. However, despite a number of papers that report a similar activity in isolated plant mitochondria (11, 29–31), a recent and more rigorous study of isolated potato mitochondria failed to find evidence of a fatty acid-stimulated proton leak that was inhibitable by nucleotides (20). This latter work casts some doubt as to whether UCP catalyzes a proton leak in plant mitochondria, although there are several different interpretations of this data. One possibility is that UCP does catalyze a proton leak, but that there is
Superoxide-stimulated Proton Leak in Potato Mitochondria

Fig. 3. Proton leak kinetics of mitochondria isolated from wild type tubers and from transgenic tubers with increased StUCP protein content. For further details see “Experimental Procedures.” Wild type (●, ■, ▲) and transgenic (○, △, ■) mitochondria were incubated with 1 mM NADH as a substrate and titrated with KCN in a medium containing 0.1% (w/v) BSA, 300 μM linoleic acid, 50 μM xanthine (●, ○), plus 0.015 unit of xanthine oxidase (●, ▲) or plus 0.015 unit of xanthine oxidase and 2 mM GTP (▲, △). Two independent transgenic lines were tested: line 18 (●–c) and line 63 (○–c). The inset in b shows the effect of addition of 24 units of superoxide dismutase prior to addition of xanthine oxidase to wild type (●) and transgenic tubers (line 18; ○). Values in all graphs are means ± S.E. of three independent experiments.

Table I

| Condition                      | Proton conductance at 130 mV (nmol of H⁺/min/mg of mitochondrial protein/mV) |
|-------------------------------|--------------------------------------------------------------------------------|
| WT                            | Line 18                                                                           | Line 63 |
| Linoleic acid + superoxide    | 1.09 ± 0.36                                                                        | 3.24 ± 0.21* |
| Linoleic acid + superoxide + GTP | 1.36 ± 0.14                                                                        | 1.18 ± 0.46 |

* Indicates significantly different from wild type (t test; p < 0.05).

Insufficient UCP protein in potato to detect this leak. On the basis of estimates of the specific activity of potato UCP when the StUCP gene is overexpressed in yeast and estimations of UCP content in potato mitochondria, this is the possibility favored by Hourton-Cabassa et al. (20). However, since isolated mitochondria do not experience the same bioenergetic conditions as mitochondria in vivo, an alternative possibility is that the conditions of assay were not sufficient to fully activate UCP.

We have shown that in the presence of exogenous superoxide, linoleic acid stimulates a proton leak in isolated potato mitochondria that is inhibited by the nucleotide, GTP (Figs. 1 and 3). This is characteristic of UCP activity and suggests that superoxide may be required for full activity of potato UCP, as is the case for mammalian UCPs (21, 22). A reactive oxygen species-dependent uncoupling of wheat mitochondria in the presence of fatty acid has previously been observed, but the nucleotide sensitivity of this effect was not tested (32). To investigate whether superoxide-dependent, fatty acid-stimulated proton conductance is indeed related to the activity of UCP, we examined the effect of increased mitochondrial UCP content in transgenic plants overexpressing the StUCP gene. Mitochondria from two independent transgenic lines contained 13-fold (line 18) and 2-fold (line 63) more UCP protein than WT (Fig. 2). This confirms that overexpression of StUCP results in a measurable increase of UCP protein and that this protein is correctly targeted to the mitochondrion. The rate of proton conductance in mitochondria isolated from these two lines was significantly higher than WT when assayed in the presence of superoxide and linoleic acid together but unaltered in the presence of linoleic acid alone (Fig. 3). This confirms that superoxide-dependent, linoleic acid-stimulated uncoupling is catalyzed by StUCP. The fact that this UCP-related uncoupling is completely inhibited by GTP (Fig. 3) provides a specific assay for UCP that can be utilized in future studies. The increase in rate of proton conductance was proportional to the increase in UCP content in one of the lines (line 63; proton conductance rate, 2.3-fold WT and UCP protein content, 2-fold WT), providing further evidence that the change in proton conductance is directly linked to UCP. However, in a second line the increase in proton conductance was much less than the increase in UCP protein content (line 18; proton conductance rate, 3.0-fold WT and UCP protein content, 13-fold WT). It is not clear why the relationship is not directly proportional in this line, although it may be related to the greater increase in UCP protein content. Previously, it has been observed that the uncoupling effect of UCP when overexpressed to very high levels is artifactual, presumably due to a misfolding of the UCP protein in the mitochondrial membrane (33). Under such circumstances it is conceivable that the direct relationship between UCP protein content and proton conductance rate may break down. How-
ever, the fact that the increased proton conductance in line 18 is completely inhibited by GTP (Fig. 3b) leads us to believe that the additional UCP in line 18 is correctly folded and inserted into the membrane (since if it were not, the resulting artifactual uncoupling would be unregulated). Alternative explanations for the lower than expected increase in proton conductance rate in this line are that some unknown endogenous factor is limiting the proton leak rate or that above 3-fold expression only some of the overexpressed UCP is inserted correctly into the membrane, while the rest has no effect on proton leak rate in these mitochondria.

This work, for the first time, shows that changes in UCP content can affect the rate of proton leak in plant mitochondria providing firm evidence that plant homologues of mammalian UCPs do function as uncoupling proteins in planta. Furthermore, we have demonstrated that xanthine/xanthine oxidase stimulates this proton leak, which suggests that superoxide is required for full activity of potato UCPs. The abolishment of the xanthine/xanthine oxidase effect by superoxide dismutase confirms that is the specific presence of the superoxide anion that is responsible for the activation of UCP. Using the conditions we have described (isolated mitochondria respiring NADH in the presence of nigericin and oligomycin) it is possible to specifically assay UCP as the superoxide-dependent, fatty acid-stimulated proton leak that is inhibited by GTP. Previously, specific assay of UCP in situ in plant mitochondria has been complicated by the possibility that other carrier proteins can also catalyze a fatty acid-dependent proton leak. Thus, nucleotide inhibition is required to demonstrate specificity of the assay for UCP. Often, ATP is used as an inhibitory nucleotide, which can cause problems in interpretation due to its content can affect the rate of proton leak in plant mitochondria.

references

1. Douce, R. (1986) Mitochondria in Higher Plants: Structure, Function, and Biogenesis, Academic Press, New York.
2. Douce, R., and Neuburger, M. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 371–414.
3. Maxwell, D. P., Wang, Y., and McIntosh, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8271–8276.
4. Möller, I. M. (2001) Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 561–591.
5. Vergerberge, U. C., and McIntosh, L. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 703–734.
6. Klingenberg, M. (1990) Trends Biochem. Sci. 15, 108–112.
7. Jezek, P., Engstova, H., Zachova, M., Vercesi, A. E., Costa, A. D. T., Arruda, P., and Garidel, K. D. (1998) Biochim. Biophys. Acta 1365, 319–327.
8. Echtay, K. S., Winkler, E., and Klingenberg, M. (2000) Nature 408, 609–613.
9. Requier, D., and Boulouard, F. (2000) Biochem. J. 345, 161–179.
10. Stuart, J. A., Cadenas, S., Jordan, M. B., Roussell, D., and Brand, M. D. (2001) Biochim. Biophys. Acta 1504, 144–158.
11. Vercesi, A. E., Martins, I. S., Silva, M. A. P., Leite, H. M. F., Cucovia, I. M., and Chalmovitch, H. (1995) Nature 375, 24.
13. Larlo, M., Klein, M., Riesmeier, J. W., Muller-Rober, B., Fleury, C., Boulouard, F., and Riquier, D. (1997) Nature 389, 135–136.
14. Watanabe, A., Nakazono, M., Totsuemi, N., and Hirai, A. (1999) Plant Cell Physiol. 40, 1160–1166.
16. Maia, I. G., Benedetti, C. E., Leite, A., Turcinielli, S. R., Vercesi, A. E., and Arruda, P. (1999) FEBS Lett. 429, 403–406.
17. Itó, K. (1999) Plant Sci. 149, 167–173.
18. Murayama, S., and Handa, H. (2000) Mol. Gen. Genet. 264, 112–118.
19. Watanabe, A., and Hirai, A. (2002) Plants 215, 90–109.
21. Jezek, P., Costa, A. D. T., and Vercesi, A. E. (1997) J. Biol. Chem. 272, 24272–24278.
22. Borecky, J., Maia, I. G., Costa, A. D. E., Jezek, P., Chalmovitch, H., de Andrade, T. C., Costa, A. D. T., Vercesi, A. E., and Arruda, P. (2000) FEBS Lett. 505, 240–244.
23. Hourot-Cabassa, C., Mesneau, A., Miroux, B., Roussau, J., Riquier, D., Zachowski, A., and Moreau, F. (2002) J. Biol. Chem. 277, 41533–41538.
24. Krysi, K. S., Murphy, M. M., Smith, R. A., Talbot, D. A., and Brand, M. D. (2002) J. Biol. Chem. 277, 47129–47135.
25. Echtay, K. S., Roussell, D., St-Pierre, J., Jakobsen, M. B., Cadenas, S., Stuart, J. A., Harper, J. A., Roebuck, J. R., Morrison, A., Pickering, S., Clapham, J. C., and Brand, M. D. (2002) Nature 415, 96–99.
26. Bevan, M. (1984) Nuclear Acids Res. 12, 8711–8721.
27. Franck, A., Guilley, H., Jonard, P., Richards, K., and Hirai, L. (1980) Cell 20, 285–292.
28. Gielan, J., De Beuckeleer, M., Steurinck, J., Dhebecque, F., De Greve, H., Lemmers, M., Van Montagu, M., and Schell, J. (1984) EMBO J. 3, 851–861.
29. Roche-Sensor, M., Sonnewald, U., Stratman, M., Schell, J., and Willmitzer, L. (1989) EMBO J. 8, 23–29.
30. Considine, M., Daley, D., and Whelan, J. (2001) Plant Physiol. 126, 1619–1629.
31. Brand, M. D. (1996) in Bioenergetics: A Practical Approach (Cooper, C., ed.) pp. 39–62, Oxford University Press, Oxford.
32. Jezek, P., Costa, A. D. T., and Vercesi, A. E. (1996) J. Biol. Chem. 271, 32743–32748.
33. Jarmuszewicz, W., Almeida, A. M., Sluse-Goffart, C. M., Sluse, F. E., and Vercesi, A. E. (1998) J. Biol. Chem. 273, 34882–34886.
34. Sluse, F. E., Almeida, A. M., Jarmuszewicz, W., and Vercesi, A. E. (1998) FEBS Lett. 435, 237–241.
35. Pastore, D., Frittianni, A., Di Pepe, S., and Passarella, S. (2000) FEBS Lett. 470, 88–92.
36. Stuart, J. A., Harper, J. A., Brindle, K. M., Jakobsen, M. B., and Brand, M. D. (2001) Biochem. J. 356, 779–789.
37. Baha, S., and Robinson, B. H. (2000) Trends Biochem. Sci. 25, 502–508.
38. Dukan, R., A-H-Mackerness, S., Hancock, J. T., and Neil, S. J. (2001) Plant Physiol. 127, 159–172.
39. Kowalski, A. J., Costa, A. D. T., and Vercesi, A. E. (1998) FEBS Lett. 425, 213–216.
40. Hanak, P., and Jezek, P. (2001) FEBS Lett. 495, 137–141.
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