Bioscience Reports, Vol. 21, No. 2, April 2001 (© 2001)

MINI REVIEW

The Discovery of an Uncoupling Mitochondrial Protein in Plants

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Received October 26, 2000

This paper describes peculiar properties of plant mitochondria and summarizes the experiments that led to the discovery of an uncoupling protein in these mitochondria. Recent advances in the study of the biochemical and physiological properties as well as on genes encoding plant uncoupling proteins are described in articles by Borecky *et al*., Jezek *et al*., and Jarmuszkiewicz *et al*. in this issue.

BACKGROUND

It is generally accepted that the electrochemical proton potential gradient ($\Delta\mu H^+$) generated by respiratory chain redox proton pumps is used by the F_1F_0-ATP synthase to produce ATP. According to the chemiosmotic theory, $\Delta\mu H^+$ can also be used to drive other energy requiring processes such as the electrophoretic exchange of matrix ATP^{4-} for cytosolic ADP^3, the electroneutral uptake of P_i, the $NADP^+$ reduction by the energy-linked transhydrogenase and the electrophoretic uptake of Ca^{2+}. the electrochemical proton potential gradient can also be dissipated as heat by the uncoupling protein (UCP) of mammalian brown fat mitochondria which are specialized for heat production [1]. Differently, a cyanide insensitive respiration pathway present in plants, fungi, trypanosomes, amoeba and other microorganisms is used to dissipate redox energy, as heat, instead of building a $\Delta\mu H^+$.

MAMMALIAN UNCOUPLING PROTEINS (UCPs)

The brown adipose tissue mitochondria (BATM) in newborn and hibernating or cold-adapted mammals possess a 32 kDa uncoupling protein (UCP) [2], that, in the presence of fatty acids, allows the protons extruded by the respiratory chain to reenter the matrix and bypass the ATP-synthase, thus permitting dissipation of $\Delta\mu H^+$ as heat, in a process called nonshivering thermogenesis. The apparently exclusive localization of UCP in BATM led previously to the proposal that it was a late evolutionary acquisition of mammals and exclusively expressed in brown adipose tissue. UCP is now abbreviated as UCP1, to distinguish it from other

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0144-8463/01/0400-0195$19.50/0

mammalian uncoupling proteins discovered in the last three years (UCP2, UCP3, BMCP1 and UCP4). Purine nucleotides such as GDP or ATP bind to an allosteric site on the protein and inhibit UCP1 [1]. The UCP1 content of BATM varies by an order of magnitude, depending on the physiological conditions, such as the thermal stress, to which the animal is exposed [1]. UCP1 is active in BATM isolated in the absence of bovine albumine (BSA), since endogenous fatty acids are essential for UCP1 activity. On the other hand, BATM can be fully coupled only in the simultaneous presence of BSA and GDP [1]. Recent results suggest that UCP1, as well as other UCPs, mediate uncoupling via a fatty acid cycling mechanism [3] in which the UCP translocates fatty acid anions outward. Subsequently, fatty acids become protonated and enter mitochondria in their protonated form, leaving the proton in the matrix [3].

CYANIDE RESISTANT RESPIRATION IN PLANT MITOCHONDRIA (AOx)

A distinct feature of plant mitochondria, in addition to their nonphosphorylating NAD(P)H dehydrogenases [4], is the presence of an alternative oxidase (AOx), [5]. AOx is a cyanide-resistant quinol oxidase sensitive to hydroxamates such as benzohydroxamate (BHAM), and represents a non-protomotive terminal oxidase that can significantly decrease proton pumping. Since AOx catalyzes the oxidation of ubiquinol to ubiquinone and the reduction of O_2 to H_2O, its activity may lead to an increased rate of respiration and the redox energy is released as heat. Together with succinate dehydrogenase (complex II) or the exogenous NAD(P)H dehydrogenases, AOx is capable of oxidizing substrates without any oxidative phosphorylation. In addition to its possible thermogenic role, AOx is important for carbohydrate oxidation since the bypassing of complexes III and IV favors the cycling of cofactors necessary for glycolysis and the Krebs cycle, which in turn maintains catabolism at a high rate and increases the synthesis of compounds necessary for growth and development [6]. Since ethylene is produced at high rates during the ripening of climacteric fruits with HCN as a biosynthesis by product, AOx has been proposed to participate in the climacteric respiratory burst because of its cyanide insensitivity.

In addition to the cyanide insensitive respiration, we observed that the rate of nonphosphorylating respiration of potato tuber mitochondria incubated in the absence of BSA was much slower after a period of ADP phosphorylation or after the addition of ATP. Figure 1 shows for comparison the rates of oxygen consumption of liver and potato isolated mitochondria supported by succinate oxidation before and after the addition of ADP in the absence of BSA. This coupling effect of ATP on the potato tuber respiring in the absence of BSA suggested that these mitochondria could possess at least one of the following inner membrane channels or pore: (I) the permeability transition pore (PTP); (II) the inner membrane anion channel (IMAC) or (III) the uncoupling protein (UCP).

MITOCHONDRIAL PERMEABILITY TRANSITION PORE (PTP)

This is characterized as a Ca^{2+}-dependent, cyclosporin A sensitive proteinaceous pore located in the inner mitochondrial membrane, whose permeability gradually

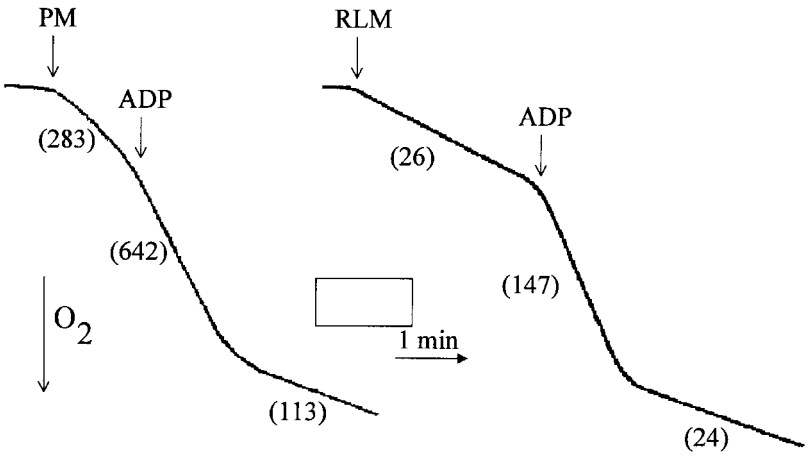

Fig. 1. Comparison of resting and phosphorylating respiration in isolated rat liver (RLM) and potato mitochondria (PM). The assay medium contained 0.25 mM sucrose, 10 mM Hepes buffer, pH 7.2, 5.0 mM P_i, 1.0 mM $MgCl^2$ and 0.5 mM EGTA. PM (0.25 mg/ml), RLM (1 mg/ml) and ADP (400 nmol) were added where indicated. Rates of respiration (nmol O/min.mg) are shown in parenthesis.

increases to larger molecules, osmotic support and even to small proteins [7]. The Ca^{2+}-induced PTP is enhanced by a variety of compounds that include inorganic phosphate, protonophores, thiol reagents, fatty acids, thyroid hormones and others [7]. In addition, to cyclosporin A, this pore can be inhibited by dithiol reductants, Mg^{2+}, EGTA and the adenine nucleotides ADP or ATP. Since we could not detect any Ca^{2+} transport activity in these mitochondria and no effect of PTP inhibitors, except of ADP or ATP, on the uncoupled respiration of potato mitochondria, we ruled out the hypothesis that this pore could explain the uncoupling in potato mitochondria.

THE INNER MEMBRANE ANION CHANNEL (IMAC)

In many respects plant mitochondria are very similar to animal mitochondria with regard to ion transport. For example, they possess the classical electroneutral anion exchange carriers for adenine nucleotides, phosphate, dicarboxilates, oxoglutyarate, tricarboxylates and pyruvate. There are also a numberof interesting differences. The relatively high permeability of plant mitochondria to H^+ and anions such as Cl^- [8] was thought to be the consequence of membrane damage associated with the isolation procedure necessary to disrupt the cell wall. However, the sustained failure to eliminate such leakages by preparing bettter coupled plant mitochondria led me to think that H^+ and anion transport in these mitochondria could be carrier mediated as in animal mitochondria. In this regard, the inner membrane of mitochondria isolated from animal tissues is normally impermeable to the electrophoretic transport of most anions at pH lower than 7.4, but this permeability progressively

increases by raising the pH to 8.8 [9]. Evidence has been provided that anion uniport in animal mitochondria is mediated by a specific pathway which is referred to as the inner membrane anion channel (IMAC). This channel is inhibited by matrix H^+, Mg^{2+}, propranolol, the alkylating agent N,N-dicyclohexylcarbodiimide, mercurials, triorganotins and nucleotides analogs such as Cibacron Blue 3 GA and Erythrosin [8]. The inactivity of IMAC in normal isolated mitochondria is a consequence of inhibition by endogenous matrix Mg^{2+} and pH.

In a collaborative work with Dr. Andrew Beavis [8] it was demonstrated that potato mitochondria contain an anion channel which is closely related to the IMAC found in animal mitochondria. Since there appeared to be significant differences between these channels, we termed the anion channel present in plant mitochondria as plant inner mitochondrial anion channel (PIMAC) [8].

During the course of these investigations on anion channel in potato mitochondria we observed that BSA also inhibited the swelling induced by nigericin when the mitochondria were suspended in KCl medium. The swelling induced by nigercin reflects that in addition to the electrophoretic pathway for the influx of Cl^-m, these mitochondria were also permeable to H^+, permitting the reentry of H^+ that had been extruded by nigericin for external K^+. Thus, the inhibition of nigericin-induced swelling by BSA suggested the existence of an endogenous protonophore activated by low concentration of fatty acids in these mitochondria. Indeed, the beneficial effect of BSA on the coupling of plant mitochondria was known for decades and strongly supported this interpretation.

UNCOUPLING PROTEIN IN PLANTS (PUMP)

We demonstrated that a fully coupled state could be reached in potato mitochondria only in the simultaneous presence of purine nucleotides such as ATP and absence of free fatty acids [10]. Thus the sequential additions of BSA and ATP were followed by the respectives increase in the transmembrane electrical potention ($\Delta\Psi$) (Figure 2). The ATP-induced change in $\Delta\Psi$, compatible with increased mitochondrial coupling, was unaffected by oligomycin, carboxyatractyloside or propranolol, inhibitors of the ATP synthase, ADP/ATP translocase and PIMAC, respectively. These results additionally suggested the existence of an H^+ conductance in potato mitochondria similar to that present in BATM. Indeed, with the help of Dr. A. C. Bianco (State University of São Paulo) we purified, in parallel experiments, the uncoupling proteins of both BAT and potato mitochondria. We called this protein plant uncoupling mitochondrial protein (PUMP) [10]. A common property of the mitochondrial P_i carrier, the ATP/ADP translocator and UCP is that these proteins are not retained in a hydroxylapatite column and at room temperature only UCP is obtained. Polyacrylamide gel electrophoresis revealed the presence of a 32KDa band both in the brown fat and in plant mitochondrial isolates. In collaboration with Drs. H. Chaimovich and I. Cuccovia (State University of São Paulo) these proteins were reconstituted in lectithin liposomes and their properties to increase an ATP or GTP-sensitive H^+ conductance of the proteoliposomes were well documented [10].

It was subsequently demonstrated that fatty acids uncoupled potato mitochondria in an ATP-sensitive manner and induced H^+ dependent mitochondrial

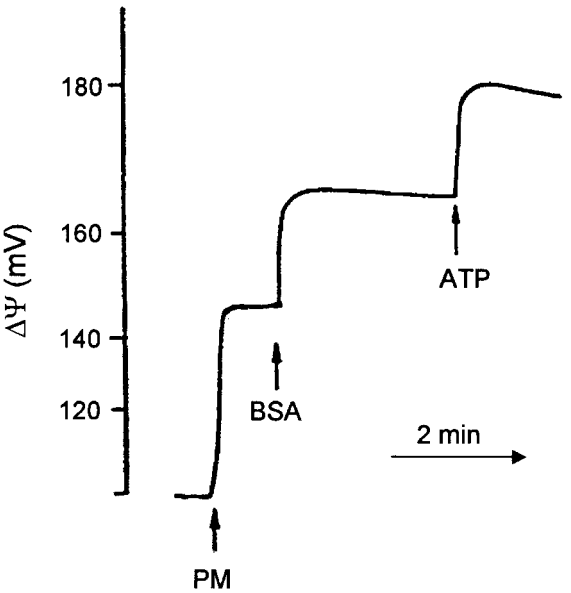

Fig. 2. Effects of ATP and BSA on transmembrane electrical potential of isolated potato tuber mitochondria. Mitochondria (1 mg protein/ml) were added to a reaction medium containing 300 mM mannitol, 20 mM KCl, 10 mM Hepes buffer, pH 7.2 and 3 μM tetraphenylphosphonium (TPP+). ATP (1.0 mM) and 0.1% BSA were added where indicated. The concentration of TPP+ in the extramitochondrial medium was continuously monitored with a TPP+-selective electrode.

swelling in K^+-acetate medium [11]. Further studies on its properties and functions demonstrated that PUMP was in all respects similar to UCP1 [11, 12], with the exception that PUMP does not translocate Cl^-.

Using antibodies raised against potato PUMP, we have immunologically detected the presence of PUMP in many climateric fruits or fruits in which an increase in respiration has been observed, thus supporting its possible participation in the climateric respiration rise. The ubiquitous character of PUMP distribution in plants suggests that it could be the counterpartner of the animal UCP2. This is supported by the higher aminoacid seqence homology of PUMP and UCP2 as compared to other UCPs (Borecky *et al.*, this issue).

Finally, we have expressed the Arabdopsis thaliana uncoupling protein gene in *Escherichia coli* and reconstituted the recombinant protein into liposomes (Borecky, J., Maia, I. G., Costa, D. T., Jezek, P., Chaimovich, H., Andrade, P. B. M., Vercesi, A. E., and Arruda, P., unpublished results). The properties of this reconstituted protein provided a definitive proof that it is an uncoupling protein and that it exhibits all the functional features of UCP2 and had the same properties of the PUMP purified from potato mitochondria. It should be stressed that PUMP was the second

uncoupling protein described, ruling out the proposition that mitochondrial uncoupling protein was a late evolutive acquisition of mammals and exclusively expressed in brown adipose tissue.

ACKNOWLEDGMENTS

The author thanks Drs. Jiri Borecky and Roger Castilho for critical reading of the manuscript and Dr. Marcia Fagian and Maria Augusta Martins for helping with the preparation of the figures. The work from the author's laboratory was supported by grants fronm the Brazilian Agencies FAPESP, CNPq/PADCT and FINEP/PRONEX.

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