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Mitochondrial Cation Transport: A Progress Report

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This report summarizes recent work in our laboratory aimed at understanding protein-mediated mitochondrial cation transport. We are studying three distinct cation cycles that contain porters catalyzing influx and efflux of cations between cytosol and mitochondrial matrix. Each of these cation cycles plays a major physiological role in the overall energy economy. The K^+ cycle maintains the integrity of the vesicular structure and includes the K^+/H^+ antiporter, the K_{ATP} channel, and K^+ leak driven by the high membrane potential. The Ca^{2+} cycle relays the signals calling for modulation of ATP production and includes the Ca^{2+} channel, the Na^+/Ca^{2+} antiporter, and the Na^+/H^+ antiporter. The H^+ cycle of brown adipose tissue mitochondria provides heat to hibernating and newborn mammals and consists of the uncoupling protein, which catalyzes regulated H^+ influx.

KEY WORDS: Mitochondria; cation; proton; transport; proteins.

INTRODUCTION

I was asked to summarize our own work, none of which would have been remotely possible without the major contributions, too numerous to cite here, made by so many mitochondrial transport laboratories around the world. These contributions are referenced in the papers cited at the end of the article.

Our research focuses on fundamental mechanisms of ion transport across membranes, with emphasis on mitochondrial cation porters. During the course of work on the K^+/H^+ antiporter, we decided that new insights into mechanism would require coupling of functional studies with site-directed mutagenesis. Both sides of this equation needed work: functional characterization had not achieved the level required for interpretation of structure-function studies, and, with the exception of the uncoupling protein, these transport proteins had not been purified and their cDNAs had not been cloned.

We embarked on a program of purification and reconstitution of the K^+/H^+ antiporter. After the major technical problems had been largely overcome, we found that our protocols were suitable for studying other cation porters, including those of the Ca^{2+} cycle and the uncoupling protein. Our criterion for successful reconstitution of transport activity has been the demonstration of activity with all the kinetic and regulatory attributes previously determined from studies in intact mitochondria. In this regard, our progress with each transporter has been satisfactory but largely technical. Nevertheless, this work is a necessary first step toward advancing the field.

To varying degrees, these reconstitution assays have also increased our fundamental understanding of each transport mechanism. Thus, we have uncovered previously unknown aspects of transport that were obscured or unmeasurable in intact mitochondria. While there is much work to do on the kinetics and regulation of these transport proteins, we are finding the reconstituted protein to be a very reliable model for such studies.

We are making slow but sure progress in purifying these proteins. This has been a daunting task for two reasons: their low abundance and their tendency to form mixed micelles with contaminating proteins. In this work, we have been encouraged by the result that polyclonal antibodies to these proteins selectively

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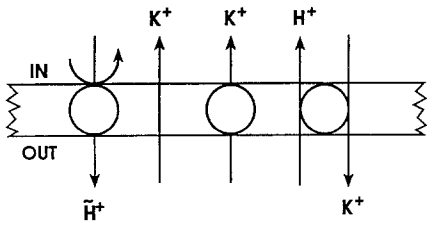

Fig. 1. The mitochondrial K^+ cycle. Electrogenic H^+ ejection by the mitochondrial redox chain drives electrophoretic K^+ uptake into the matrix by parallel leak and channel pathways. Internal K^+ is then released in exchange for protons via the electroneutral K^+/H^+ antiporter, which is dynamically regulated by matrix free Mg^{2+} ions. The K^+ cycle is a futile cycle required for physiological control of matrix volume. (From Paucek *et al.*, 1992, with permission.)

inhibit transport. These antibodies have been used to screen cDNA libraries, and in two cases partial clones of 1.6 and 2.3 kb appear to encode the K^+/H^+ antiporter and K_{ATP} channel, respectively.

THE VOLUME-REGULATORY POTASSIUM CYCLE OF MITOCHONDRIA

From its very beginning, the chemiosmotic theory encompassed more than the mechanism of biological energy conservation. Mitchell recognized that the postulated protonic batteries would generate transmembrane forces that would have serious consequences for mitochondrial physiology within the cell. Thus, the membrane potential required to drive ATP synthesis would cause cations, particularly potassium, to leak in through the coupling membrane, accompanied by anions and water and threatening swelling and lysis. Because operating $\Delta\Psi$ is so high, cation uptake must be balanced by extrusion of ions against the electrical gradient. In recognition of this physiological necessity, he postulated the existence of ion exchange carriers for anions and cations (Mitchell, 1961, 1966).

In particular, maintenance of mitochondrial integrity necessitates that net K^+ flux remains zero in the face of rapid fluxes through independent pathways (Fig. 1) (Garlid, 1980; Paucek *et al.*, 1992). Steady-state volume regulation appears to be mediated primarily by the K^+/H^+ antiporter, which is inhibited reversibly by internal Mg^{2+} ions in a manner that is exquisitely sensitive to volume changes and excessive uptake of K^+ and anions (Garlid, 1988). The K^+/H^+ antiporter is an 82-kDa inner membrane protein (Martin *et al.*, 1984) and was the first cation porter purified and reconstituted in our laboratory (Kakar *et al.*, 1989; Jezek *et al.*, 1990a; Li *et al.*, 1990). Antibodies to this protein were found to inhibit transport, and antibody screening has yielded a 1.6-kb cDNA fragment (Sun *et al.*, 1994). Antibodies purified to the fusion protein expressed by this fragment recognize the purified 82-kDa band on Western blot and strongly inhibit transport. The existing fragment contains at least three membrane-spanning domains and a putative nucleotide-binding site at the 3′ end. Preliminary studies of nucleotide inhibition of the reconstituted beef heart K^+/H^+ antiporter indicate that ADP inhibits ($K_i \approx 200\,\mu M$) whereas ATP does not (Sun *et al.*, 1994). If this observation is verified, it represents a new mechanism of volume regulation and may explain an important observation by Chavez *et al.* (1977) that $^{42}K^+/K^+$ exchange in beef heart mitochondria was markedly inhibited after ADP was added to initiate state 3 respiration.

It is now established that mitochondria contain a K_{ATP} channel (Paucek *et al.*, 1992). Because it operates in parallel with K^+ leak (Garlid *et al.*, 1989), it is difficult to study in intact mitochondria. Nevertheless, both light scattering (Beavis *et al.*, 1993) and patch clamp (Inoue *et al.*, 1991) confirm that intact mitochondria contain such a process. We have partially purified this protein and, in collaboration with Mironova, have demonstrated that this K^+ channel is the same that she had characterized electrophysiologically (Mironova *et al.*, 1981), although it was not previously recognized as a K_{ATP} channel. The following summarizes some characteristics of this channel as revealed by electrophoretic K^+ flux studies in liposomes reconstituted with the liver and heart K_{ATP} channel and containing the fluorescent probe PBFI to follow K^+ transport.

Perhaps the most remarkable characteristic of the mitochondrial K_{ATP} channel is that it exhibits all the salient properties of the family of plasma membrane K_{ATP} channels: It conducts K^+ ions selectively, and Na^+ is neither transported nor does it affect K^+ flux. It is inhibited by ATP ($K_i \approx 60\,\mu M$) and glibenclamide with high affinity ($K_i \approx 50$ nM). In the presence of ATP, it is activated by other nucleotides and by K_{ATP} channel openers, including diazoxide and chromakalim. Gauthier and Diwan (1979) had shown that a component of mitochondrial K^+ flux is inhibited by DCCD (dicyclocarbodiimide), and we have found DCCD to be a potent, irreversible inhibitor of mitochondrial K_{ATP} channels. We have reconstituted the cardiac sarcolemmal K_{ATP} channel (Paucek and

Garlid, 1993) and have found that it, too, is inhibited 100% by DCCD treatment. Thus, the mitochondrial K_{ATP} channel exhibits a rich panoply of ligand interaction and clearly belongs to the family of K_{ATP} channels (Paucek *et al.*, 1992).

Just as plasma membrane channels possess tissue-specific variations with respect to regulation, the mitochondrial K_{ATP} channel also has its own personality. For example, Mg^{2+} is absolutely required for ATP inhibition, whereas Mg^{2+} has no effect on ATP inhibition of the sarcolemmal K_{ATP} channel. The physiological imperative for regulation of the mitochondrial K_{ATP} channel, which will dissipate energy and uncouple oxidative physphorylation, is satisfied by its high affinity for ATP. On the other hand, this feature raises the question: How can it be opened under physiological conditions? We have obtained new results that partially answer this question. Both GTP ($K_a \approx 7\,\mu M$) and GDP ($K_a \approx 100\,\mu M$) are able to fully activate the mitochondrial K_{ATP} channel in the presence of $500\,\mu M$ ATP, a dose at which K^+ flux is otherwise 100% inhibited.

A major challenge to bioenergetics is to uncover the physiological role of the mitochondrial K_{ATP} channel. Two possibilities suggest themselves. (i) The K^+ channel may open up to expand matrix volume during mitochondrial biogenesis and in association with synthesis of proteins and expansion of membranes. Volume expansion would require net K^+ uptake, mediated by an imbalance between activity of the ATP-dependent K^+ channel and the K^+/H^+ antiporter. (ii) Regulated energy dissipation through operation of the K^+ channel and the K^+/H^+ antiporter may be necessary during periods of low cellular requirements for ATP in order to maintain flow and metabolism of reducing equivalents between matrix and cytosol.

We have made some progress toward identifying the K_{ATP} channel protein and its cDNA. We raised polyclonal antibodies to a partially purified, 54-kDa protein and found that these antibodies are potent inhibitors of K^+ flux through the reconstituted K_{ATP} channel. The antibodies were used to screen a cDNA library and yielded several positive clones. Antibodies were purified to the fusion proteins expressed in bacteria, and those purified to one clone inhibited K^+ flux with greater affinity than the parent antibody. Controls did not inhibit. These results are encouraging, but much work is yet required to sequence the cDNA and verify that this clone encodes the mitochondrial K_{ATP} channel.

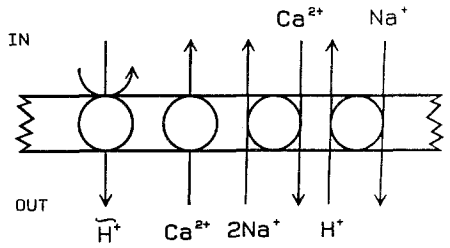

Fig. 2. The mitochondrial Ca^{2+} cycle. Electrogenic ejection of two protons drives electrophoretic uptake of one Ca^{2+} via the inner membrane Ca^{2+} channel. Internal Ca^{2+} is then released in exchange for two Na^+ via the electroneutral Na^+/Ca^{2+} antiporter, and the Na^+ is then released via the electroneutral Na^+/H^+ antiporter. The Ca^{2+} cycle is a futile cycle required for physiological control of ATP production. (From Li *et al.*, 1992, with permission.)

THE METABOLISM-REGULATORY Ca^{2+} CYCLE

The mitochondrial Ca^{2+} cycle is designed to regulate intramitochondrial Ca^{2+} levels and to relay changes in cytosolic $[Ca^{2+}]$ to the mitochondrial matrix. Free matrix $[Ca^{2+}]$, in turn, regulates key matrix enzymes. Surges in cytosol $[Ca^{2+}]$ activate cellular, ATP-requiring processes via a number of second messenger systems. The emerging consensus is that this message is transmitted to the mitochondrial matrix via the Ca^{2+} transport cycle so that the level of oxidative phosphorylation can be attuned to increasing cellular demands for ATP (Denton and McCormack, 1985; Hansford, 1985).

The inner membrane contains at least three porters for regulation of mitochondrial $[Ca^{2+}]$. The Ca^{2+} channel is characterized by high-capacity, electrophoretic Ca^{2+} uptake. Free mitochondrial $[Ca^{2+}]$ would exceed cytosol levels *in vivo* by 10^6 if mitochondrial Ca^{2+} were at equilibrium with respect to Ca^{2+} uniport at 180 mV, but instead mitochondrial and cytosolic Ca^{2+} levels are comparable. Clearly, a Ca^{2+} efflux mechanism is required for removal of Ca^{2+} in the face of a high electric field. The electroneutral Ca^{2+} efflux mechanism consists of two porters whose net result is to exchange one matrix Ca^{2+} for two cytosolic protons. The inward Na^+ gradient drives Ca^{2+} efflux via the electroneutral Na^+/Ca^{2+} antiporter, and Na^+ is removed by the electroneutral Na^+/H^+ antiporter. The energy expenditure of the futile Ca^{2+} cycle (Fig. 2) is the cost of regulating mitochondrial metabolism to meet the energetic needs of the cell.

We obtained a highly purified, reconstitutively active fraction containing the mitochondrial Ca^{2+}

channel (Zhou *et al.*, 1993), using the ethanol extraction protocol of Mironova *et al.* (1982). Fura-2 was used to measure Ca^{2+} uptake driven by a K^+ gradient in the presence of valinomycin. The K_m for Ca^{2+} ranged between 7 and 20 μM, and the V_{max} was 130 μmol/mg protein/min, approximately 250-fold greater than that observed in intact mitochondria. La^{3+} exhibited competitive inhibition of Ca^{2+} uptake, and ruthenium red exhibited noncompetitive inhibition with an IC_{50} of 2–3 nM. Ca^{2+} transport in the reconstituted system exhibited hyperbolic dependence on $[Ca^{2+}]$, in contrast to its behavior in intact mitochondria, where Ca^{2+} appears to be an allosteric activator of transport (Gunter and Pfeiffer, 1990). This finding raises the interesting possibility that Ca^{2+} activation of Ca^{2+} uniport in intact mitochondria may be mediated by a separate regulatory protein.

We have identified and purified the reconstitutively active, 59-kDa Na^+/H^+ antiporter (Garlid *et al.*, 1991). Polyclonal antibodies raised to this protein are potent inhibitors of Na^+ flux (Shariat-Madar and Garlid, 1993); however, the antisera also contains antibodies to the 60-kDa mitochondrial chaperonin. We have recently separated these two proteins and have begun to purify antibodies in preparation for cDNA library screening.

We purified the Na^+/Ca^{2+} antiporter from beef heart mitochondria and reconstituted it into liposomes containing the ion-selective fluorescent probes Fura-2 or SBFI (Li *et al.*, 1992). Measurements of Ca^{2+} uptake and Na^+ efflux at pH 7.3 each yielded K_m values for Ca^{2+} of 1 μM. *Cis* K^+ activated Na^+/Ca^{2+} exchange by lowering the K_m for Ca^{2+} to 0.3 μM. The K_m for Na^+ uptake was 7 mM. Na^+/Ca^{2+} exchange is obligatorily electroneutral, as demonstrated by the finding that it occurs spontaneously in liposomes. Electrophoretic exchange would require a counterion. Furthermore, Na^+ efflux was approximately twice Ca^{2+} influx. To our surprise, the Na^+/Ca^{2+} antiporter was found to catalyze high rates of Na^+/Li^+ and Na^+/K^+ exchange when Ca^{2+} was excluded from the medium, and both exchanges were inhibited completely by diltiazem and tetraphenylphosphonium. Diltiazem and tetraphenylphosphonium cation inhibited Na^+/Ca^{2+} exchange with K_i values of 10 μM and 0.6 μM, respectively. Na^+/Ca^{2+} exchange was very sensitive to pH, with maximal activity at pH 7.3, in general agreement with findings in intact mitochondria by Baysal *et al.* (1991). The effect of pH was mediated entirely via an effect on the K_m for Ca^{2+}. The V_{max} for Na^+/Ca^{2+} exchange was increased 6- to 8-fold by bovine serum albumin ($K_a \approx 25$ nM), an effect that appears not to be due to removal of fatty acids and may reflect masking of an autoregulatory domain in the carrier protein, as observed with the plasma membrane Na^+/Ca^{2+} exchanger (Kleiboeker *et al.*, 1992). The kinetic and regulatory parameters obtained on the purified, reconstituted Na^+/Ca^{2+} antiporter are consistent with a central role in the regulation of matrix Ca^{2+} activity.

We have obtained polyclonal antibodies that inhibit reconstituted Na^+/Ca^{2+} exchange. During the course of cDNA screening, we discovered that the antibodies, as well as the 110-kDa protein fraction, contain significant amounts of transhydrogenase, and we have only recently succeeded in separating this contaminant.

THE THERMOGENIC UNCOUPLING PROTEIN

The uncoupling protein (UCP) is the exception that proves the rule of the chemiosmotic theory, in that its function is to short-circuit the protonic batteries, thereby causing mitochondria to produce heat instead of ATP. UCP is uniquely expressed in one mammalian organ, the thermogenic brown adipose tissue. In addition to its own attributes, it is of interest because it belongs to the same gene family as the Pi/H symporter, the adenine nucleotide translocase and the oxoglutarate carrier.

Uncoupling protein mediates electrophoretic transport of protons and anions across the inner membrane of brown adipose tissue mitochondria. The mechanism and site of proton transport, the mechanism by which fatty acids activate proton transport, and the relationship between fatty acids and anion transport have been the subject of intense speculation, but remained unknown. UCP has generally been considered to be a H^+ or OH^- uniporter. We felt that anion transport was the key to mechanism, both because it lacked obvious physiological significance and because it was difficult to integrate with H^+ transport. We used fluorescent probes to measure H^+ and anion transport in vesicles reconstituted with purified uncoupling protein (Jezek *et al.*, 1990b) and developed a new method for measuring H^+ flux in liposomes (Orosz and Garlid, 1993). We found a new class of transported anions, the alkylsulfonates, whose flux increased with hydrophobic chain length (Jezek *et al.*, 1990c). Accordingly, we hypothesized

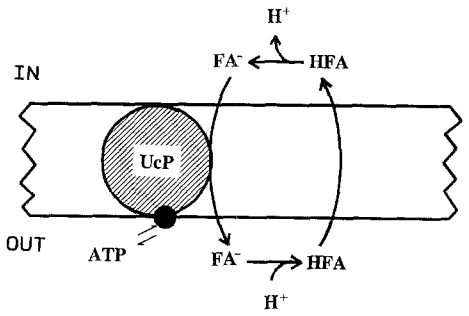

Fig. 3. The mitochondrial H^+ cycle. Electrogenic H^+ ejection by brown adipose tissue mitochondria drives electrophoretic efflux of fatty acid anions (FA^-) through uncoupling protein (UCP). The futile cycle is completed by rapid nonionic diffusion of the protonated fatty acids into the matrix. UCP thus enables fatty acids, whose anions are poorly permeable, to behave as cycling anionic protonophores. This H^+ cycle is utilized by mammals for heat production.

that the energy well for anion flux is identical with the fatty acid docking site in the protein (Garlid, 1990).

A comparative study of the effects of laurate and its close analogue, undecanesulfonate, revealed that undecanesulfonate was transported by uncoupling protein with a K_m value similar to that observed for laurate as it activated H^+ transport. Both laurate and undecanesulfonate inhibited Cl^- with competitive kinetics. Undecanesulfonate inhibited laurate-induced H^+ transport with competitive kinetics. Laurate differed from undecanesulfonate in two important respects: (i) Laurate caused electrophoretic, uncoupling-protein-mediated H^+ transport, whereas undecanesulfonate was incapable of inducing H^+ transport. (ii) Lauric acid was rapidly transported across the bilayer by nonionic diffusion, whereas undecanesulfonic acid was not transported at all. We infer that fatty acids induce H^+ transport *because* they can diffuse electroneutrally across the membrane. The role of uncoupling protein in H^+ transport is therefore to transport fatty acid anions, enabling them to behave as cycling anionic protonophores (Fig. 3). According to this hypothesis, originally proposed by Skulachev (1991), uncoupling protein is a pure anion porter and does not transport protons (Modriansky *et al.*, 1994).

UCP has been cloned, and we have developed a very useful protocol for its heterologous expression in yeast (Murdza-Inglis *et al.*, 1991). Levels of expression are about as high as in native brown adipose tissue mitochondria, nearly 10% of yeast mitochondrial protein. We have been able to purify and reconstitute expressed UCP following site-directed mutagenesis. Our most promising result to date has been that mutation of a single arginine in the putative nucleotide-binding domain to leucine completely abolished GDP inhibition of fatty acid-induced H^+ transport without affecting the transport itself (Murdza-Inglis *et al.*, 1994).

Assuming that the proposed mechanism for UCP holds up to scrutiny, we are left with a physiological conundrum. Our work (unpublished data) has shown that there is no interaction between Cl^- or fatty acids and GDP inhibition. That is, GDP is a purely noncompetitive inhibitor of transport through UCP. This means that neither ATP nor fatty acids can regulate uncoupling. Analogous to the K_{ATP} channel, it is easy to see how UCP can be closed, but it is difficult to understand how it opens, given its high affinity for ATP. What is needed is an activator that either displaces or reduces ATP without inhibiting. Some years ago, Cannon *et al.* (1977) proposed palmitoyl CoA for this role, and our laboratories are collaborating in a reinvestigation of this and other possibilities for cellular regulation of UCP.

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