The Inner Mitochondrial Membrane Has Aquaporin-8 Water Channels and Is Highly Permeable to Water*

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Mitochondria are remarkably plastic organelles constantly changing their shape to fulfil their various functional activities. Although the osmotic movement of water into and out of the mitochondrion is central for its morphology and activity, the molecular mechanisms and the pathways for water transport across the inner mitochondrial membrane (IMM), the main barrier for molecules moving into and out of the organelle, are completely unknown. Here, we show the presence of a member of the aquaporin family of water channels, AQP8, and demonstrate the strikingly high water permeability (Pf) characterizing the rat liver IMM. Immunoblotting, electron microscopy, and biophysical studies show that the largest mitochondria feature the highest AQP8 expression and IMM Pf. AQP8 was also found in the mitochondria of other organs, whereas no other known aquaporins were seen. The osmotic water transport of liver IMM was partially inhibited by the aquaporin blocker Hg²⁺, while the related activation energy remained low, suggesting the presence of a Hg²⁺-insensitive facilitated pathway in addition to AQP8. It is suggested that AQP8-mediated water transport may be particularly important for rapid expansions of mitochondrial volume such as those occurring during active oxidative phosphorylation and those following apoptotic signals.

Mitochondrial volume is of pivotal importance for the activity of the electron transport chain (1) and a control point of apoptosis (2). Changes in mitochondrial volume occur in many other physiological and patho-physiological conditions, including intracellular signal transduction, liver regeneration, ischemia/reperfusion-induced damage, and axoniasis (3–5). Mitochondria are well behaved osmometers, and swelling and contraction of the mitochondrial matrix and related changes to mitochondrial morphology are the consequence of the water movement that osmotically accompanies the net transport of solutes into and out of the mitochondrion (6), respectively. Mitochondrial volume changes are modulated by the net movement of solutes including K⁺ and Ca²⁺ ions across the IMM1 (7, 8). The inner mitochondrial membrane acts as a major barrier for the solutes and water moving between the cytoplasm and the mitochondrial matrix, the outer membrane being freely permeable for molecules of up to 1.5 kDa due to the presence of the exceedingly large pores formed by VDAC, the voltage-dependent anion channel (9). However, although a number of IMM transport systems have been cloned and characterized for their ability to transport solutes across the IMM (10), the molecular pathway for the movement of water remains obscure. Important clues for understanding the molecular bases of the mitochondrial osmotic properties were recently provided by the identification of an aquaporin water channel (11), AQP8, in rat hepatocyte mitochondria (12). AQP8 was also found in intracellular vesicles that are shuttled to the hepatocyte apical membrane under choleretic stimuli such as those brought about by glucagon (13). This led us to hypothesize the existence of two distinct pools of AQP8 in hepatocytes, one involved in primary bile secretion and another related to intracellular osmoregulation (14). As AQP8 is predominantly expressed within the cell interior (12, 15) and displays a very distinct gene organization and evolutionary pathway (16, 17), we also suggested that AQP8 evolved separately to feature intracellular specializations lacking in other mammalian aquaporins (12).

To investigate possible correlations between mitochondrial AQP8 expression, and mitochondrial morphology and water permeability, we have now defined the biochemical and ultrastructural localization of AQP8 in the submitochondrial compartments, characterized the molecular pathways and biophysics of water transport across the IMM, and suggested functions for AQP8 in mitochondria.

MATERIALS AND METHODS

Subcellular and Submitochondrial Membrane Fractionations—Subcellular membranes were prepared from adult male Wistar rats weighing 250–300 g (Morini, S. Polo D’Enza, Italy). Rats were fed with a standard diet and water ad libitum. For all experiments, rats were decapitated after anesthesia, and livers and other organs were quickly removed and processed depending on the specific preparation. All experiments carried out were in accordance with the principles for research involving animals authorized by The University of Bari. For the isolation of subcellular membrane fractions, livers were homogenized with a Potter-Elvehjem homogenizer (four strokes in 1 min at 500 rpm) in an isolation medium consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, and 5 mM EGTA, pH 7.4. The homogenate was centrifuged at 500 × g for 10 min at 4 °C, and the pellet consisting of nuclei and unbroken cells was discarded; the resulting supernatant was centrifuged at 1,000 × g for 10 min at 4 °C and the related pellet was treated with 0.25 M sucrose to preserve subcellular membrane fractions, which were subsequently centrifuged at 20,000 × g for 20 min at 4 °C.

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‡ The abbreviations used are: IMM, inner mitochondrial membrane; AQP, aquaporin; OMM, outer mitochondrial membrane; PBS, phosphate-buffered saline.
(1,000 × g membrane fraction) was washed twice before being resuspended in isolation medium to which a mixture of protease inhibitors had been added (1 mM PMSF, 1 mM leupeptin, 1 mM pepstatin A). The 1,000 × g supernatant was collected, centrifuged at 3,000 × g for 10 min at 4 °C, and the resulting pellet washed twice leading to the 3,000 × g membrane fraction. A similar procedure was used to prepare the 6,000 × g and 17,000 × g fractions of subcellular membranes. The preparation of the outer mitochondrial membrane (OMM) and mitoplasts was performed by using the detergent approach described by Ragan et al. (18). Briefly, digitonin (Calbiochem) was added to a suspension of 1,000, 3,000, or 6,000 × g mitochondria (100 mg of protein/ml) to a final concentration of 0.6% (w/v) in isolation medium and incubated for 15 min on ice under gentle stirring. After dilution with 3 volumes of isolation medium, the suspension was centrifuged at 15,000 × g for 10 min at 5 °C. The resulting pellet (mitoplasts) was saved, whereas the supernatant was centrifuged at 144,000 × g for 20 min at 4 °C leading to a pellet (outer mitochondrial membrane fraction), which was then washed twice. The protein concentration was assayed by the Lowry method. The relative purity of the outer membrane and mitoplasts was assessed from the specific activity of marker enzymes (monoamine oxidase and malate dehydrogenase for the outer membrane and mitoplasts, respectively) as described previously by Ragan et al. (18). All chemicals used for the preparations except digitonin were from Sigma.

**Inner Mitochondrial Membrane Vesicle Preparation**—The IMM vesicles were prepared as reported previously (18) from the 1,000, 3,000, or 6,000 × g subpopulation of rat liver mitochondria suspended at a protein concentration of 100 mg/ml. Briefly, mitoplasts were prepared as above and resuspended in isolation medium at a protein concentration of 15 mg/ml before being sonicated with a probe sonicator (Vibra-Cell, Sonics & Materials Inc., Berlin, Germany) for six 5-s bursts at the maximum energy setting with 30-s cooling periods. After sonication, mitoplasts were diluted with an equal volume of isolation medium and centrifuged at 15,000 × g for 10 min at 5 °C. The resulting pellet was resuspended in 10 volumes of isolation medium and centrifuged again at 100,000 × g; this process was repeated twice. The final pellet was resuspended in isolation medium to which protease inhibitors had been added (1 mM PMSF, 1 mM leupeptin, 1 mM pepstatin A) and protein content was assessed by assaying the cytochrome c oxidase. The diameter of the IMM vesicles was determined both by a particle size analyzer and by electron microscopy (see below).

**Immunoblotting Analysis**—Aliquots (60 μg of proteins) of isolated mitochondria, outer membrane, or mitoplasts prepared as above were heated to 90 °C and electrophoresed in an SDS, 12% acrylamide gel (Mighty Small II, Amersham Biosciences) using a low molecular weight marker ladder (Amersham Biosciences, Buckinghamshire, UK). The resolved proteins were transferred electrophoretically onto nylon membranes that were blocked in 5% (w/v) low fat milk in blocking buffer (20 mM Tris-HCl, 0.15 mM NaCl, 1% Triton X-100, pH 7.5) for 1 h, and further incubated with an affinity-purified rabbit antibody directed against an N-terminal rat AQP8 (J. H. Pfister, generously donated by Couler Inc., Palo Alto, CA) and by morphometric analysis of electron micrographs. The time course of vesicular volume change was followed from changes in intensity of scattered light at the wavelength of 450 nm using a Jasco FP-6200 (Jasco, Tokyo, Japan).Stopped-flow reaction analyzer that has a 1.6-ms dead time and 99% mixing efficiency in <1 ms. The sample temperature was controlled by a circulating water bath. To perform the experiments, 35 μl of a concentrated vesicle suspension was diluted into 2.5 ml of a hypotonic (220 mosM) isolation medium (124 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, and 5 mM EGTA, pH 7.4). One of the syringes of the stopped-flow apparatus was filled with the vesicle suspension, whereas the other was filled with the same buffer added of mannitol to reach a final osmolality of 500 mosM to establish a hypertonic gradient (140 mosM) upon mixing. The final protein concentration after mixing was 100 μg/ml. Immediately, after applying a hypertonic gradient, water outflow occurs, and the vesicles shrink, causing an increase in scattered light intensity. The data were fitted to a single exponential function. The osmotic water permeability coefficient (Pf), an index reflecting the osmotic water permeability of the vesicular membrane, was calculated as described (19), using the equation: \( P_f = K_w \Delta V / \Delta C \), where \( K_w \) is the fitted exponential rate constant, \( \Delta V \) is the initial mean of vesicle volume, \( \Delta C \) is the molar volume of water, and \( \Delta C \) is the osmotic gradient. The medium osmolality was verified by freezing point depression, using a Halbmiro-Osmometer (Knauer, Berlin, Germany).

In some experiments, IMM vesicles were incubated for 5 min in isolation medium deprived of EGTA and EDTA and containing 300 μM of nenedylyl compound known to block mitochondrial inhibitors including AQP8 (20). In other experiments, to verify the blocking action of the Hg\(^{2+}\) ion, the HgCl\(_2\) treatment of the vesicles was followed by a 15 min exposure to 10 mM of the reducing agent β-mercaptoethanol. The temperature dependence of osmotic water permeability in the IMM vesicles in the presence or absence of Hg\(^{2+}\) inhibition was assessed by determining the activation energy of Arrhenius (\( \mathcal{E}_a \)) calculated measuring the \( P_f \) of the vesicles at 10, 20, and 30 °C.

**RESULTS AND DISCUSSION**

As a first step, we defined the precise subcellular localization of AQP8 in rat liver. This was done by immunohistochemistry and immunoblotting using an affinity-purified immunoglobulin G specific for the rat AQP8 polypeptide. Strong immunohistochemical AQP8 reactivity was seen within the hepatocyte cell interior (Fig. 1A). Overall, AQP8 labeling was found to be greater in the periporal and midlobular than pericentral regions of the hepatic lobule, although hepatocytes with heavy immunostaining were sometimes found in the pericentral region (Fig. 1A, red block arrow). For the immunoblotting experiments, the AQP8 antibody was incubated with fractions of rat liver membranes prepared at different gravitational forces as reported previously (21). A band of expected size, 28 kDa, was strongly detected in the 1,000 and 3,000 × g fractions and,
Supporting a predominant expression of AQP8 in intracellular organelles, the 28-kDa band was very weak in the plasma membrane-enriched fraction (17,000 × g) (Fig. 1B). Interestingly, after normalizing for the different percentage of mitochondrial composings the gravitational pellets, densitometric analysis of the above immunoblots showed that the heavier the mitochondrial fraction the stronger the intensity of the 28-kDa band (Fig. 1D). By electron microscopy analysis, the average mitochondria size in the 1,000 × g liver fraction was almost twice and three times larger than the 3,000 and 6,000 × g fractions, respectively (1.18 ± 0.4, 0.68 ± 0.4, and 0.45 ± 0.3 μm, respectively). Suggesting a wide expression in mitochondria, AQP8 immunoreactivity was detected in the mitochondrial fractions of kidney (Fig. 1E) and many other rat organs known to express such AQP including testis, heart, duodenum, jejunum, and colon (data not shown). AQP8 was apparently absent in epididymal spermatozoa (data not shown). Control immunoblotting using antibodies against AQP9, an aquaporin reported to be expressed only in the hepatocyte sinusoidal membrane (22), showed the absence of immunoreactivity in the mitochondrial fractions, while a band of expected molecular mass, 29 kDa, was detected in the fractions containing plasma membrane (Fig. 1F). Localization of AQP8 in the inner mitochondrial membrane was indicated by immunoblotting experiments showing higher AQP8 immunoreactivity in mitoplasts than whole mitochondria and negligible staining in the OMM (Fig. 1G). This finding was fully consistent with studies of immunogold electron microscopy with both in situ and freshly isolated mitochondria, in both cases showing AQP8 immunoreactivity in the IMM and, occasionally, the matrix compartment (Fig. 2, A, B, and D). No distinctive labeling was observed in the OMM when this membrane was clearly visible (Fig. 2D). Morphometric analysis of freshly isolated mitochondria (Fig. 2F) showed variable AQP8 reactivity among mitochondria, some of which appeared poorly labeled or even unreactive. This pattern of distribution is consistent with the heterogeneous distribution of AQP8 in liver (Fig. 1A) and suggests the existence of subpopulations of mitochondria where AQP8 is not expressed. This possibility is corroborated by the apparent absence of immunogold AQP8 particles in the mitochondria of rat epididymal spermatozoa (data not shown). Interestingly, such mitochondria have the distinctive metabolic feature of not performing the β-oxidation of fatty acids and to use fructose as the only carbon source. Future studies will be addressed to identify the metabolic features of the subpopulations of mitochondria expressing AQP8. Consistent with a possible involvement of AQP8 in rapid increases in mitochondrial volume, swollen mitochondria showed AQP8 immunostaining in the IMM (Fig. 2C). Confirming the wide presence of AQP8 in mitochondria, immunogold particles were seen in in situ mitochondria of rat testis (Fig. 2D). No mitochondrial labeling was seen in control experiments where mitochondria were incubated with antibodies directed against the other hepatocyte aquaporin, AQP9 (data not shown).

Having found the presence of an aquaporin water channel in the IMM, we then decided to characterize the biophysical properties of mitochondrial osmotic water transport. To investigate the reciprocal contribution of the channel-mediated (facilitated diffusion) and lipid bilayer (simple diffusion) pathways to the movement of water through the IMM, we directly assessed osmotic water permeability ($P_o$) and related activation energy ($E_a$) of basically pure and homogeneous IMM vesicles by measuring scattered light intensity using stopped-flow spectrophotometry. Mitochondria and mitoplasts could not be used for the IMM $P_o$ calculation as they are of heterogeneous size (Fig. 2, A–D) and irregular shape (Fig. 3A), respectively. IMM vesicles
from the 1,000, 3,000, or 6,000 \times g subpopulations of intact mitochondria were obtained by sonication from liver mitoplasts and had a mean vesicle diameter of 234 \pm 31 (n = 626), 248 \pm 36 (n = 611), and 243 \pm 28 (n = 676) nm, respectively (Fig. 3B).

Vesicles were subjected rapidly to a hypertonic osmotic gradient (140 mosM), and the time course of the vesicle shrinkage was followed from the change in scattered light. The osmotic membrane water permeability of all three subpopulations of IMM vesicles was strikingly high (Fig. 3, C and D) in line with the rapid changes in volume which mitochondria undergo during metabolic homeostasis (23). The highest values of \( P_f \) were obtained with the IMM vesicles prepared from the heaviest mitochondria, since the \( P_f \) values were of 524.5 \pm 6.7, 496.6 \pm 9.0, and 341.4 \pm 4.5 \mu m/s for the 1,000, 3,000, and 6,000 \times g fraction, respectively (Fig. 3D). Interestingly, the \( P_f \) values paralleled the expression rates of AQP8 in the same mitochondrial populations from which the IMM vesicles were prepared (Fig. 1, A and B). Proving the absence of mixing artifact, no change in scattered light was observed when vesicles were mixed with isosmotic buffer (Fig. 3C).
creases in the rate of osmotic water transport was observed when the vesicles were subjected to the same osmotic gradient (140 mosM) at increasing temperatures. Arrhenius plotting of the calculated $P_f$ values at 10, 20, and 30 °C resulted in an $E_a$ of 3.93, 4.51, and 4.93 kcal/mol for the 1,000, 3,000, and 6,000 × g IMM vesicles, respectively. These $E_a$ values, all biophysically consistent with water permeation through aqueous channels (19), showed only slight increases when the IMM vesicles were exposed to HgCl$_2$ before the stopped-flow light scattering measurements (4.71, 4.86, and 5.11 kcal/mol for the 1,000, 3,000, and 6,000 × g IMM vesicles, respectively). Although the Hg$_{2}^{2+}$-inhibitable route accounts for most water permeability of the IMM vesicles, respectively. Overall, as well as biophysically characterizing the osmotic movement of water across the IMM is postulated and deserves investigation. AQP8-mediated water movement may be particularly important in the homeostatic control of mitochondrial volume such as in the swelling that the mitochondrion undergoes under apoptotic stimuli and during oxidative phosphorylation. Recognition of AQP8 in mitochondria therefore has profound new implications for an understanding of how mitochondria adapt their morphology.

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