Evidence against Functionally Significant Aquaporin Expression in Mitochondria*

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Recent reports suggest the expression of aquaporin (AQP)-type water channels in mitochondria from liver (AQP8) (Calamita, G., Ferri, D., Gena, P., Liquori, G. E., Cavalier, A., Thomas, D., and Svelto, M. (2005) J. Biol. Chem. 280, 17149–17153) and brain (AQP9) (Amiry-Moghaddam, M., Lindland, H., Zelenin, S., Roberg, B. A., Gundersen, B. B., Petersen, P., Rinvik, E., Torgner, I. A., and Ottersen, O. P. (2005) FASEB J. 19, 1459–1467), where they were speculated to be involved in metabolism, apoptosis, and Parkinson disease. Here, we systematically examined the functional consequence of AQP expression in mitochondria by measurement of water and glycerol permeabilities in mitochondrial membrane preparations from rat brain, liver, and kidney and from wild-type versus knock-out mice deficient in AQPs -1, -4, or -8. Osmotic water permeability, measured by stopped-flow light scattering, was similar in all mitochondrial preparations, with a permeability coefficient \( P_f \sim 0.009 \text{ cm/s} \). Glycerol permeability was also similar \((\sim 5 \times 10^{-6} \text{ cm/s})\) in the various preparations. \( \text{HgCl}_2 \) slowed osmotic equilibration comparably in mitochondria from wild-type and AQP-deficient mice, although the slowing was explained by altered mitochondrial size rather than reduced \( P_f \). Immunoblot analysis of mouse liver mitochondria failed to detect AQP8 expression, with liver homogenates from wild-type/AQP8 null mice as positive/negative controls. Our results provide evidence against functionally significant AQP expression in mitochondria, which is consistent with the high mitochondrial surface-to-volume ratio producing millisecond osmotic equilibration, even when intrinsic membrane water permeability is not high.

Functionally significant expression of aquaporin (AQP)-type water channels has been demonstrated in plasma membranes in various cell types in kidney, brain, eye, glandular epithelia, endothelia, epidermis, fat, and other tissues. As demonstrated by phenotype comparisons of wild-type and AQP-deficient mice, plasma membrane AQPs are important in the urinary concentration mechanism, glomerular fluid secretion, regulation of intraocular and intracranial pressures, cell migration, brain swelling, epidermal hydration, adipocyte metabolism, and other functions (1). Whether AQPs play a role in intracellular organelar functions is less clear. The vasopressin-regulated water channel AQP2 is expressed in both the plasma membrane and in a recycling endosomal compartment in kidney collecting cells (2, 3). Although AQPs in the kidney collecting duct are highly water-permeable (4), it is likely that their high water permeability is a consequence of dense AQPs expression rather than a need for high endosomal water permeability. Indeed, the high surface-to-volume ratio of endosomes and other organelles, generally \( >3 \times 10^{-2} \text{ cm}^3 \) (diameter \(< 200 \text{ nm}\) predicts very rapid osmotic equilibration times of \(<100 \text{ ms}\), even when osmotic water permeability \( (P) \) is low \(($<0.005 \text{ cm/s}$)\). Other AQPs have also been reported in endosomes in some cell types, including AQP6 in the renal proximal tubule and collecting duct epithelial cells (5) and various AQPs in liver cells (6), although their possible functions are not well characterized.

Two recent studies reported AQP expression in mitochondria and suggested possible involvement of mitochondrial AQPs in many functions, including metabolism and apoptosis, and in the pathogenesis of neurological diseases such as Parkinson disease. Calamita et al. (7) report AQP8 expression in rat mitochondria with high water permeability that was partially inhibited by \( \text{HgCl}_2 \). Amiry-Moghaddam et al. (8) report AQP9 expression in astroglia throughout rat brain and in a subset of neurons, although no functional analysis was done. As speculated in these reports, AQP expression and function in mitochondria could have wide-ranging biological consequences. However, our recent phenotype analysis of AQP8 knock-out mice shows normal liver phenotype (9), and a preliminary analysis of recently generated AQP9 knock-out mice by the Nielsen laboratory (10) did not demonstrate authentic AQP9 immunoreactivity in mouse brain, suggesting that prior AQP9 immunoreactivity in brain may have been artificial.

Because of the potential importance of mitochondrial AQP expression, we have systematically examined the predicted functional consequences of such expression. We have focused on functional transport measurements of mitochondrial inner membrane preparations rather than on antibody staining studies because of the relatively poor available antibodies against AQP8 and AQP9. AQP8 function was studied by comparative measurements of osmotic water permeability in liver mitochondrial membranes from wild-type versus AQP8 knock-out mice, as well as from \( \text{HgCl}_2 \) effects and water permeability measurements in mitochondrial membranes from several different mouse and rat tissues. AQP9 function was studied by measurements of water and glycerol permeabilities in brain mitochondria, as AQP9 is an efficient glycerol transporter (11, 12).

**MATERIALS AND METHODS**

**Animals**—Adult wild-type mice and transgenic mice in a CD1 genetic background lacking AQP1, AQP4, or AQP8 protein were generated by targeted gene disruption as described previously (9, 13, 14). Adult female Sprague-Dawley rats were purchased from Charles River Laboratories. All animal procedures were approved by the University of California-San Francisco Committee on Animal Research.

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2 The abbreviations used are: AQP, aquaporin; IMM, inner mitochondrial membranes.
Mitochondria Isolation and Preparation of Inner Mitochondrial Membranes (IMMs)—Tissues were homogenized with a Potter-Elvehjem homogenizer in isolation medium consisting of 220 mm mannitol, 70 mm sucrose, 20 mm Tris-HCl, 1 mm EDTA, 5 mm EGTA, 20 mm phenylmethylsulfonyl fluoride, 1 mm leupeptin, and 1 mm pepstatin A, pH 7.4, at 4 °C as described previously (7). The homogenate was centrifuged at 500 × g for 10 min at 4 °C. The pellet consisting of nuclei and unbroken cells was discarded and the supernatant was centrifuged at 1,000 × g for 10 min at 4 °C, washed twice, and resuspended in isolation medium. The 1,000 × g supernatant was collected, centrifuged at 3,000 × g for 10 min at 4 °C, washed twice, and resuspended. A similar procedure was used to prepare the 6,000 and 17,000 × g fractions. IMMs were prepared using a detergent approach as described previously (7). Briefly, digitonin was added to suspensions of 1,000, 3,000, or 6,000 × g fractions (100 mg of protein/ml) separately 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 three volumes of isolation medium, the suspension was centrifuged at 15,000 × g for 10 min at 4 °C. The resulting pellet (mitoplasts) was resuspended in isolation medium at 15 mg protein/ml and sonicated with a probe sonicator for 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 4 °C. The resulting pellet was resuspended in 10 volumes of isolation medium and centrifuged again at 100,000 × g; this process was repeated twice. Vesicle purity was determined by assay of cytochrome c oxidase and alkaline phosphodiesterase I activities as described previously (15).

Vesicle Size Measurement—Vesicle diameter was determined by quasi-elastic light scattering using a Zetasizer (Malvern Instruments, Inc., Southborough, MA). For electron microscopy, suspended mitoplasts or IMM vesicles were fixed in 1% glutaraldehyde in 47 mM sodium cacodylate at pH 7.4 for 5 min and then centrifuged at 13,000 × g for 10 min. The pellet was post-fixed in 1% veronal acid for 1 h and dehydrated in graded ethanol and embedded in araldite. Sections of ~80 nm thickness were cut and photographed on a JEOL 1200 EX electron microscope operating at 80 kV.

Immunoblot Analysis—Total protein was assayed in subcellular fractions using a commercial assay (DC protein assay kit, Bio-Rad, Richmond, CA) and loaded on a 12% SDS-polyacrylamide gel (10 μg/lane). Proteins were blotted to polyvinylidene difluoride membranes (Gelman Scientific, Ann Arbor, MI) and immunoblotted by standard procedures. Membranes were incubated with 1:1000 dilution of rabbit polyclonal serum raised against an N-terminal peptide (NH2-SMDLEPKVKTSMAGRC-COOH) of mouse AQP8 (generated by Abgent, San Diego, CA).

Stopped-flow Measurement of Water and Glycerol Permeabilities—Stopped-flow measurements were done on a Hi-Tech SF-51 instrument. For measurement of osmotic water permeability, vesicle suspensions were mixed in under 1 ms with a hypertonic mannitol solution (in the same buffer as the samples, but with 300 mm mannitol added). The kinetics of decreasing mitochondria/vesicle volume was measured from the time course of 90° scattered light intensity at a 530-nm wavelength. Osmotic water permeability coefficients (P) were calculated as described previously (16). In some experiments, samples were incubated with 0.3 mm HgCl2 (prepared freshly) for 5 min prior to stopped-flow measurements. For measurement of glycerol permeability, mitochondria were subjected to a 150 mM inwardly directed gradient of glycerol. Glycerol permeability (Pglycerol) was computed as Pglycerol = 1/[S/V]τ, where S/V is surface-to-volume ratio (assuming smooth, spherical vesicle) and τ is the exponential time constant fitted to the vesicle swelling phase of the light scattering time course corresponding to glycerol entry.

RESULTS

Characterization of Mitochondrial Preparations—Mitochondrial membrane fractions and IMMs were isolated from mouse and rat liver. Fig. 1A shows morphology of the 3,000 × g fractions (whole mitochondria, top; IMM, bottom) from the livers of wild-type and AQP8 null mice. The size distribution of whole mitochondria and IMM of the 3,000 × g fraction determined by quasi-elastic light scattering are shown in Fig. 1B. The electron microscopy and quasi-elastic light scattering data show reasonably uniform size distributions from which average diameters were deduced for computation of permeability coefficients. Fig. 1C verifies mitochondrial purity as judged by the activities of the mitochondrial marker with cytochrome c oxidase and the plasma membrane marker alkaline phosphodiesterase I. By immunoblot analysis, a 28-kDa band representing non-glycosylated AQP8 was found in liver homogenate and plasma membranes (17,000 × g fraction) from wild-type mice (Fig. 1D). A very weak band was seen in the 3000 × g fraction, likely repre-
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FIGURE 2. Osmotic water permeability in inner mitochondrial membranes from liver. A, representative curves for the time course of scattered light intensity at 10 °C in response to a 150 mM inwardly directed gradient of mannitol. Data shown for IMM (1,000, 3,000, and 6,000 × g fractions) from wild-type and AQP8 null mice and from rat. Vesicle diameters (in nm) were 298 ± 25, 285 ± 18, and 193 ± 42 (1,000, 3,000, and 6,000 × g fractions from wild-type mice); 302 ± 11, 297 ± 24, and 283 ± 27 (AQP8 null mice); 317 ± 26, 307 ± 22, and 302 ± 29 (rat). B, Pf (mean ± S.E., three preparations) computed from experiments as for A. Differences were not significant. C, same as for A, except at 25 °C. D, Arrhenius plots of ln Pf versus reciprocal absolute temperature (1/T).

senting plasma membrane contamination. No specific AQP8 band was seen in liver homogenate from AQP8 null mice.

Water Permeability Measurements—Water permeability in liver IMM vesicles (3,000 × g fraction) from wild-type and AQP8 null mice and from rats was measured by stopped-flow light scattering in response to a 150 mM inwardly directed gradient of mannitol. Scattered light intensity is inversely related to vesicle volume. Fig. 2A shows representative light scattering data at 10 °C, a low temperature in which AQP-facilitated water permeability would be best seen. The kinetics of osmotic equilibration of each of the three IMM vesicle fractions was similar. Fig. 2B summarizes water permeability coefficients (Pf) deduced from the kinetic data and IMM size. Pf values were similar for each of the IMM fractions from mice and rats and not reduced by AQP8 deletion. Temperature dependence measurements were done to investigate the mechanism of IMM water transport. Fig. 2C shows light scattering data at 25 °C. The water permeability in each vesicle fraction was strongly temperature-dependent, increasing ∼2.2-fold from 10 to 25 °C. Fig. 2D summarizes the data as an Arrhenius plot of ln Pf versus reciprocal absolute temperature, where the slope is proportional to the activation energy. Activation energy was computed to be 9–10 kcal/mol, suggesting a lipid diffusion rather than an aquaporin-facilitated mechanism for water transport.

Incubation of liver IMM with HgCl2 slowed by ∼2-fold the kinetics of osmotic equilibration in IMM from mouse and rat liver, including IMM from AQP8 null mice (Fig. 3A). The HgCl2 effect is thus not related to AQP8 but could be due to a different mitochondrial HgCl2-sensitive water pathway or to a change in vesicle surface-to-volume ratio or aggregation, which could change the kinetics of osmotic water equilibration in the absence of altered intrinsic membrane water permeability. Fig. 3B shows that apparent vesicle diameter increased by ∼1.7-fold after HgCl2 incubation. Computed Pf values, which take into account vesicle size, were not significantly reduced by HgCl2 (Fig. 3C).

Osmotic water permeability measurements were done on mitochondria from different organs, each organ expressing a different aquaporin (liver, AQP8; brain, AQP4; kidney, AQP1; heart, AQP8) (Fig. 4A). Measurements were done on mitochondria from wild-type mice and from mice lacking the appropriate AQP, as well as on rats. Fig. 4B summarizes the Pf values determined for each of the preparations from the data in Fig. 4A and mitochondrial diameters determined by quasi-elastic light scattering. There was no significant difference in the Pf values in the various mitochondrial preparations, suggesting absence of functionally significant AQP expression in mitochondria.

Glycerol Permeability Measurements—To test for AQP9-facilitated glycerol transport in rat brain mitochondria, glycerol permeability was compared in mitochondria from rat brain, liver, and kidney. Glycerol permeability was measured by light scattering following a 150 mM inwardly directed gradient of glycerol. Fig. 5A shows representative light scattering data, with the slow decrease in scattered light intensity corresponding to glycerol influx into mitochondria. Experiments were done at 10 °C (left panel) and 25 °C (right panel).
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was no significant difference in glycerol permeability coefficients ($P_{\text{glycerol}}$) in the brain versus liver and kidney (Fig. 5B). Fig. 5C summarizes the data as an Arrhenius plot of $\ln P_{\text{glycerol}}$ versus reciprocal absolute temperature. Activations energies were in the range 16–18 kcal/mol, suggesting lipid- rather than aquaporin-facilitated glycerol transport.

**DISCUSSION**

The purpose of this study was to test the hypothesis that AQP8 and AQP9 provide quantitatively important pathways for osmotically induced water movement across inner mitochondrial membranes. As described in the introduction, these experiments were motivated by recent reports of AQP8 expression in liver mitochondria (7) and AQP9 expression in brain mitochondria (8). These reports proposed a novel paradigm for aquaporin function with significant implications for mitochondrial biology. The predictions of functionally significant AQP-dependent mitochondrial water permeability include mitochondrial volume regulation during active oxidative phosphorylation (7) and adult neural stem cell differentiation (17). Amiry-Moghaddam et al. have hypothesized that altered mitochondrial AQP9 in dopaminergic neurons may relate to their vulnerability in Parkinson disease (8). Our study here has defined the functional consequence of AQP expression in mitochondria from measurements of water and glycerol permeabilities in mitochondrial membrane preparations from brain, liver, and kidney in rats and in wild-type versus knock-out mice deficient in various aquaporins.

AQP8 is expressed in multiple organs (9, 18–23). The liver is a major site of AQP8 transcript expression in rat and mouse (6, 9, 19–21, 24). However, conflicting data have been reported for the subcellular localization of AQP8 expression in liver, perhaps because of the poor available AQP8 antibodies. Immunohistochemistry in rat shows AQP8 protein expression in intracellular vesicles in hepatocytes (21, 25). Garcia et al. (26) report a cAMP agonist-induced relocalization of intracellular AQP8 to the plasma membrane in rat hepatocytes. Ferri et al. (24) report AQP8 expression in intracellular membranes in mouse liver, including smooth endoplasmic reticulum, subapical vesicles, and mitochondria. Utilizing a mouse AQP8 antibody and liver from a AQP8 knock-out mouse as the negative control, we have shown AQP8 protein expression on the plasma membrane with weak intracellular localization (9). Immunoblot data here showed AQP8 immunoreactivity in liver homogenate and the plasma membrane (17,000 $\times$ g fraction) but not in mitochondrial membranes.

Water permeability was compared in mitochondria from wild-type versus AQP8 null mice to define the functional expression of AQP8 in mitochondria. Data from IMM and whole mitochondria showed a high Arrhenius activation energy (9–10 kcal/mol) and a relatively low osmotic water permeability (0.009 cm/s at 10°C), suggesting lipid- rather than aquaporin-facilitated water transport. It is difficult, however, to interpret absolute $P_0$ values in mitochondria in terms of the presence of AQP8s because of the higher water permeability of intracellular, cholesterol-poor membranes compared with cholesterol-rich plasma membranes and because of uncertainties in membrane infoldings and, hence, the validity of the assumption that the mitochondrial vesicles are smooth spheres. In contrast to our measurements here, Calamita et al. (7) report a substantially lower activation energy (3.9–4.9 kcal/mol) in rat mitochondria. They also report higher $P_0$ values of up to 0.05 cm/s, which may be related to their measurements being done at a higher temperature, to differences in IMM size, and/or to the use of an exponential approximation in the computation of $P_0$, which is not valid for large osmotic gradients.

We conclude that the rapid volume equilibration in mitochondria in response to an osmotic gradient is due to its small size (high surface-to-volume ratio) rather than to high intrinsic membrane water permeability. Further, water permeability was similar in mouse and rat mitochondria from different organs and not affected by the deletion of organ-specific aquaporins (AQP4 in brain; AQP1 in kidney, AQP8 in heart). HgCl$_2$ slowed the kinetics of osmotic equilibration comparably in mitochondria from wild-type and AQP8

**FIGURE 4. Osmotic water permeability of mitochondria from brain, liver, kidney, and heart.** A, whole mitochondria (3,000 $\times$ g fraction) from wild-type mice (first column), various AQP knock-out mice (as labeled, second column), and rat (third column) were subjected to a 150 mM inwardly directed gradient of mannitol at 10°C. B, summary of $P_0$ values (mean $\pm$ S.E., three preparations). Differences not significant.

**FIGURE 5. Glycerol permeability of mitochondria from rat brain, liver, and kidney.** A, representative light scattering data for whole mitochondria (3,000 $\times$ g fraction) subjected to a 150 mM inwardly directed gradient of glycerol at 10°C (left) and 25°C (right). The initial increase in light scattering results from osmotic water efflux (vesicle shrinkage), followed by a slower decrease caused by glycerol entry. B, $P_{\text{glycerol}}$ at 10°C (mean $\pm$ S.E., three preparations). Differences not significant. C, Arrhenius plot of $\ln P_{\text{glycerol}}$ versus reciprocal absolute temperature (1/1) for inner membrane vesicles from brain, liver, and kidney.
null mice as well as rat. However, the slowed osmotic equilibration could be accounted for quantitatively by altered mitochondrial size rather than reduced intrinsic membrane water permeability. Together, these data provide direct evidence against aquaporin-facilitated water transport in mitochondria.

AQP9, an aquaglyceroporin that transports water and glycerol, was reported to be expressed in rat brain mitochondria (8). Osmotic water and glycerol permeabilities in mitochondria from various rat tissues were compared as a test for functional AQP9 expression in rat brain mitochondria. We did not measure glycerol permeability in brain mitochondria from mice because of recent data suggesting the absence of AQP9 protein in mouse brain (10). Permeabilities from rat brain mitochondria were compared with those from rat kidney and liver mitochondria, the latter organs not expressing an aquaglyceroporin. Neither water nor glycerol permeabilities differed in mitochondria from the various tissues, with the low glycerol permeability coefficient and strong temperature-dependence suggesting lipid-mediated glycerol permeability.

In summary, the results here provide functional evidence against a role for aquaporins in mitochondria. Although we believe it unlikely, the possibility cannot be ruled out that non-transporting spliced variants of aquaporins might be present in mitochondria and be involved in non-transporting functions.

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