Reconstitution of water channel function of aquaporins 1 and 2 by expression in yeast secretory vesicles

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1Laboratory of Epithelial Cell Biology, Renal Electrolyte Division, Department of Medicine, University of Pittsburgh Medical Center, Pittsburgh 15213-2500; 3Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260; and 3Departments of Biological Chemistry and Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185

COURY, Larry A., JOHN C. Mathai, G. V. Ramesh Prasad, Jeffrey L. Brodsky, Peter Agre, and Mark L. Zeidel. Reconstitution of water channel function of aquaporins 1 and 2 by expression in yeast secretory vesicles. Am. J. Physiol. 274 (Renal Physiology 43): F34–F42, 1998.—Aquaporins 1 (AQP1) and 2 (AQP2) were expressed in the yeast secretory mutant sec6-4. The mutant accumulates post-Golgi, plasma membrane-targeted vesicles and may be used to produce large quantities of membrane proteins. AQP1 or AQP2 were inducibly expressed in yeast and were localized within isolated sec6-4 vesicles by immunoblot analysis. Secretory vesicles containing AQP1 and AQP2 exhibited high water permeabilities and low activation energies for water flow, indicating expression of functional AQP1 and AQP2. AQP1 solubilized from secretory vesicles was successfully reconstituted into proteoliposomes, demonstrating the ability to use the yeast system to express aquaporins for reconstitution studies. The AQP2-containing secretory vesicles showed no increased permeability toward formamide, urea, glycerol, or protons compared with control vesicles, demonstrating that AQP2 is highly selective for water over these other substances. We conclude that the expression of aquaporins in yeast sec6 vesicles is a valid system to further study mammalian water channel function.

activation energy; heterologous protein expression; proton permeability; water and solute permeability

WATER CROSSES MEMBRANES by diffusion through either the lipid bilayer or through specific water channels (aquaporins; reviewed in Refs. 3 and 52). Water permeability is ~20-fold greater when water channels are expressed in oocytes or reconstituted (3, 52), and water permeation occurs with an activation energy of 2–5 kcal/mol (25) instead of the activation energy of 10–15 kcal/mol observed for diffusion across a lipid bilayer. Six aquaporins (AQP0–AQP5) have been cloned from mammals. AQP0 [major intrinsic protein (MIP)] is located in lens fiber cells (40, 48). AQP1 is found in the renal proximal tubule (9), red blood cells, and a variety of endothelia (30, 52). AQP2 mediates vasopressin-stimulated water flow across the apical membrane of kidney collecting duct cells, whereas both AQP3 and AQP4 are constitutively expressed and allow water flow across the basolateral membrane of these cells (18, 52). AQP5 is localized in salivary and lacrimal glands, cornea, and lung (38). Although the physiological significance of these proteins is not completely defined, loss of function mutations in AQP2 result in severe nephrogenic diabetes insipidus (7, 8), and mutations in AQP0 in mice lead to cataract formation (42). However, extremely rare individuals with AQP1 mutations have been identified and lack a significant clinical phenotype (37).

Aquaporins are members of the larger MIP family of proteins based on sequence homology (33). Although >80 proteins have been cloned from the MIP family, few have been biophysically characterized (3, 52), so it is difficult to define the structure and function of the water pores formed by the individual aquaporins. Most studies of aquaporin function have involved expression in Xenopus oocytes (7, 10, 12, 35, 36). Reconstitutions of AQP1 protein into proteoliposomes have provided direct measurements of water permeability (49, 50). Data from reconstitution studies may be difficult to obtain due to lack of purified protein, and it is not always clear that the functional properties of the native protein are maintained during its solubilization and insertion into the bilayer (48). Only AQP1 from red blood cells (49, 50) and AQP0 from the bovine eye lens (31, 48) are easily purified in sufficient quantities from tissues for reconstitution and biophysical studies. Studies in oocytes are limited by a lack of biophysical definition because the interior environment cannot be modified and because endogenous channels may produce spurious activities. Oocytes have at times produced conflicting biophysical results, such as in the case of AQP3 (10, 17, 24), and, in some cases, results in oocytes (26) have conflicted with those obtained from reconstitution into planar lipid bilayers (13, 31). Thus an expression system is needed in which unique aquaporin cDNAs may be expressed. Vesicles containing the aquaporins from the expression system may then be studied under conditions in which the composition of the solutions on both sides of the channel can be controlled.

To this end, we now present the expression of AQP1 and AQP2 in the well-characterized sec6-4 yeast expression system to examine their biophysical properties. Aquaporins were expressed in yeast, and secretory vesicles were isolated. The temperature-sensitive sec6-4 mutant accumulates plasma membrane-targeted vesicles upon shift to the nonpermissive temperature (37°C) and has previously been used to study other membrane proteins (19, 28, 39). Although a previously published study showed functional expression of AQP1 in yeast secretory vesicles, the water permeability increased only two- to fourfold (21), indicating that the
system would not be useful for detailed studies of the biophysical properties of aquaporins. In the present study, conditions for aquaporin expression were optimized so that water permeabilities up to ~60-fold higher than control vesicles were obtained. From secretory vesicles containing AQP1, successful reconstitution into proteoliposomes was demonstrated. Moreover, the selectivity properties of AQP2 were defined.

MATERIALS AND METHODS

Materials. All reagents were of the highest purity available. Common reagents were from Sigma (St. Louis, MO), C. J. T. Baker (Phillipsburg, NJ), or Bio-Rad (Hercules, CA). 5,6-Carboxyfluorescein (CF) was from Molecular Probes (Eugene, OR). Affinity-purified antibodies to AQP1 and AQP2 were generated and characterized as described previously (18, 50).

Yeast strains and plasmid construction. SY1 (34) was the yeast strain used for all work. cDNAs encoding the aquaporin proteins were cloned into the yeast expression vector pYES2 (Invitrogen, San Diego, CA) by standard techniques (41). The human AQP1 cDNA was cloned into the HindIII and BamH I sites of the pYES2 vector, the human AQP2 cDNA (provided by Peter Deen and Carel van Os, University of Nijmegen, The Netherlands) was cloned into the EcoRI site, and the orientation was verified by digestion with Kpn I. Yeast was transformed by electroporation with a Bio-Rad gene pulser with the following settings: 1.5 kV, 200 ohms, and 25 μF. Transformed yeast was grown and maintained in defined media lacking uracil and containing raffinose as a carbon source. Yeast was transferred to rich yeast extract-peptone (YPE)-galactose medium (0.5% yeast extract, 1% bactopeptone, and 2% galactose) at 25°C for 2-4 h to initiate protein expression and then was switched to 37°C overnight to force accumulation of the secretory vesicles. Control studies were performed using vesicles prepared from the background strain or from yeast transformed with the pYES2 vector lacking any aquaporin insert. Both sets of control vesicles gave identical results in permeability studies.

Immunoblotting. Samples were incubated at 60–80°C for 5 min and then separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 4–20% continuous-gradient Tris(hydroxymethyl)aminomethane (Tris)-Cl-glycine Ready Gels or 12% SDS-polyacrylamide slabs (20) and transferred to nitrocellulose (Bio-Rad). The blots were blocked with blot buffer consisting of 1% powdered milk and 3% Tween 20 in phosphate-buffered saline (pH 7.25). The blots were incubated with 1:2,000 affinity-purified, anti-AQP1 (43) or anti-AQP2 (29) antibody overnight at 4°C and then visualized by the enhanced chemiluminescence method (NEN or Amersham).

Vesicle preparation. Vesicles were prepared as described previously (6, 28) but modified for stopped-flow studies. Briefly, growth conditions were modified as described above, the yeast was treated with 10 mM dithiothreitol (DTT) in 100 mM Tris-Cl, pH 9.4, and spheroplasts were generated by digesting the cell wall with bacterially expressed recombinant lyticase. The plasma membrane was cross-linked with concanavalin A to increase its density above that of the secretory vesicles. Spheroplasts were lysed in lysis buffer (0.8 M sorbitol, 10 mM triethanolamine, 1 mM EDTA, pH 7.2) containing CF (7.5 mg/ml), and unlysed cells and concanavalin-cross-linked plasma membranes were pelleted at 11,000 revolutions/min (20,000 g) in a Sorvall GSA rotor for 10 min at 4°C. Vesicles were pelleted from the supernatant and washed to remove extravesicular CF by centrifugation at 29,000 revolutions/min (144,000 g) in a Sorvall TH-641 swinging-bucket rotor for 1 h at 4°C. The surface area-to-volume ratio was calculated as previously described (51).

Reconstitution. Secretory vesicles were solubilized in 1.5% (wt/vol) octyl glucoside, 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.5, 20% glycerol, 1 mM DTT, 0.4% E. coli phospholipids, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) for 20 min on ice and centrifuged at 100,000 g for 1 h at 4°C. The supernatant was used for reconstitution into proteoliposomes (4). Octyl glucoside extract of secretory vesicles (5 mg/ml) was solubilized in 5 mg of bath-sonicated E. coli phospholipid containing 1.5% (wt/vol) octyl glucoside. This mixture was incubated on ice for 20 min. Proteoliposomes were formed by rapid dilution of the mixture into 25 ml of 150 mM N-methyl glucamine, 50 mM MOPS, pH 7.5, 15 mM CF, 1 mM DTT, and 0.5 mM PMSF (buffer A) at room temperature. Proteoliposomes were pelleted by centrifugation for 1 h at 100,000 g at 4°C. The pellet was resuspended in 300 μl of buffer A lacking CF as previously described (50). Protein concentrations were measured as described previously (4).

Water, solute, and proton transport. Water transport was measured by stopped-flow fluorescence quenching as previously described (22, 49, 50). Briefly, the integrity of the yeast vesicles that were loaded with CF during lysis was verified by first measuring their fluorescence intensity on an SLM-AMINCO SPF-500C spectrophotometer connected to an NEC/MultiSync 2A computer. Extravesicular CF was then quenched by addition of an anti-CF antibody, and vesicles were shrunk by successive additions of a high-osmolal solution using sucrose as the osmotant. Vesicle shrinkage correlated linearly with the osmolality increase over the range of values tested. Yeast vesicles were loaded onto an Applied Photophysics SF.7mv stopped-flow apparatus with a measured dead time of 0.7 ms. The vesicles were subjected to an abrupt doubling of the osmolality, causing the vesicles to shrink and the CF to self-quench. Water permeabilities were calculated using MATHCAD software as previously described (22, 49, 50). For solute and proton permeabilities, the vesicles were rapidly subjected to a solute gradient (700 mM inside, 300 mM outside) or pH gradient (pH 6.8 inside, pH 5.0 outside). All experiments were designed to force a decrease in fluorescence, either due to shrinkage and CF self-quenching or due to the pH sensitivity of CF fluorescence. The decrease in fluorescence was measured and correlated to the decrease in volume. Fluorescence was measured with incident light of 490 ± 1 nm and a cut-off filter that measures light emitted at wavelengths >510 nm.

RESULTS

Whether from control, AQP1-, or AQP2-expressing yeast, isolated secretory vesicles behaved as a single population with a median diameter of 188 ± 13 nm. This value did not change appreciably for the different vesicle preparations and was used for all permeability calculations. This is somewhat larger than the 100-nm yeast secretory vesicle size reported from electron microscopy measurements within whole yeast cells (16) and may reflect changes in vesicle size during preparation.

As shown in Fig. 1, AQP1 and AQP2 vesicles, but not control vesicles, contained detectable aquaporin proteins by immunoblotting. Each lane contains total vesicle protein either from an aquaporin-expressing strain or from a control strain transformed with the
pYES vector lacking aquaporin. To determine if the yeast expression system produced functional protein, both AQP1-containing and AQP2-containing sec vesicles were assayed for osmotic water permeability (Pf). As shown in Fig. 2, Pf increased dramatically in AQP1-containing vesicles compared with control. Note the difference in time scale along the abscissa. The Pf for the control vesicles was 0.0022 ± 0.0004 (SD) cm/s (n = 5). The original Pf value obtained for AQP1-containing vesicles was 0.030 ± 0.010 cm/s (n = 3). Expression conditions were optimized, and water permeability measurements reached values as high as 0.087 cm/s. Figure 2 shows the rate of vesicle shrinkage after optimization for expression.

When AQP1 was solubilized from the yeast secretory vesicles and reconstituted into artificial proteoliposomes (49, 50), the water permeability increased proportionately with the amount of AQP1 protein (Fig. 3). Control proteoliposomes contained protein from yeast secretory vesicles lacking AQP1 at a protein-to-lipid ratio of 1:1, whereas the other graphs show protein additions from AQP1-containing yeast. Protein from AQP1-expressing yeast was reconstituted at total protein-to-lipid ratios of 1:1, 1:2, and 1:5, with increasing amounts of protein leading to increased rates of water flux. Thus the fast shrinkage rate of the AQP1-containing proteoliposomes was due specifically to the AQP1 protein. Further evidence for this is shown in Fig. 4, which shows the differences in activation energy between AQP1-containing and control proteoliposomes. The proteoliposomes had activation energies of 3.7 kcal/mol (AQP1 proteoliposomes, protein/lipid = 1:1) and 12.1 kcal/mol (control proteoliposomes containing protein from SY1 yeast, protein/lipid = 1:1). These results demonstrate that the yeast system can serve as a source of aquaporin protein for reconstitution studies.

The human AQP2 cDNA was also expressed in yeast sec vesicles. Sec vesicles isolated from this AQP2-containing strain showed a dramatic increase in water permeability (Fig. 5). Vesicle shrinkage was complete in ~4 ms after rapid exposure to hypertonic solution. The rate of water flow in AQP2-containing vesicles may have been faster than that of AQP1-containing vesicles because of a higher protein expression level. The Pf for the AQP2-containing vesicles was 0.14 ± 0.03 cm/s (n = 4; P < 0.001). The activation energy for water transport in the AQP2-containing vesicles was 4.0 ± 0.5 kcal/mol compared with 13.2 ± 1.2 kcal/mol for control secretory vesicles (Fig. 6). The activation energy for AQP2-containing vesicles was measured over a smaller temperature range because the trapped CF leaked, possibly due to lysis, from the vesicles at higher temperatures.

AQP1 has been shown to be highly selective for water over other substances, including urea, glycerol, and protons (49, 50). Some aquaporins, such as AQP3 and the glycerol facilitator of yeast, are known to mediate fluxes of small nonelectrolytes, such as glycerol and urea, in addition to water (10, 17). Some evidence has
been presented to suggest that AQP1 and AQP2 can also mediate such fluxes when expressed in oocytes (1, 2). To determine the selectivity for water of AQP2, the permeabilities to formamide, urea, and glycerol were measured (Figs. 7–9). In all three cases, water fluxes were measured in the vesicles used for solute flux studies (see Figs. 7–9, insets). Despite the 64-fold increase in water permeability mediated by expression of AQP2, there were no detectable differences in flux rates of formamide, urea, or glycerol in control vesicles or vesicles containing AQP2. Solute permeability values for all preparations are summarized in Table 1 in which means ± SE are reported for three to five separate secretory vesicle preparations; only water permeability was significantly increased (P < 0.001) in vesicles with AQP2.

The selectivity of AQP2 for water over protons was also determined because evidence from Ussing chamber studies as well as measurements of proton flux across endosomes had indicated that a closely related aquaporin, the aquaporin responsible for vasopressin-mediated water flow in toad bladder, conducts protons (14, 15). Figure 10 shows the rate of change of internal pH when sec vesicles at pH 6.8 were rapidly subjected to an external pH of 5.0. The change in fluorescence and change in pH are linearly related over this range. Despite the 64-fold increase in Pw for AQP2-containing vesicles over control vesicles, the rate of proton flux was unaltered, demonstrating its high degree of selectivity for water over protons.

DISCUSSION

Although numerous animal and plant aquaporins have been cloned and identified, there is still little understanding of how these unique proteins form water channels (3, 52). There are several aspects of aquaporin function which, if correlated with primary structure, would provide insights into how the protein functions. These include the unit conductance, the selectivity for water over other substances, the ratio of osmotic to diffusive water permeability (which defines the length of the single-file water pore), and the ability of different solutes to penetrate partially into the water pore.

The presently known aquaporins have been expressed in Xenopus oocytes, from which some informa-
tion about their function has been obtained. However, oocytes do not permit detailed biophysical definition of aquaporin function because the solution on the cytoplasmic side of the aquaporin cannot be controlled. In addition, it is possible that oocytes alter aquaporin expression at the membrane or function in a manner distinct from regulation in vivo (7).

Reconstitution of aquaporins into proteoliposomes allows detailed biophysical definition of aquaporin function. However, large quantities of protein must be isolated, thereby reducing the numbers of aquaporins that can be studied. Furthermore, mutant forms of the proteins are difficult to obtain. In addition, solubilization and reconstitution of aquaporins may alter their function. This appears to occur with MIP (AQP0), which, when reconstituted into planar bilayers, creates a high-conductance, low-selectivity ion channel (48). It has been reported that MIP reconstituted into proteoliposomes will permit large molecules, such as glucose, to permeate the vesicles (13, 31). By contrast, when MIP is expressed in oocytes, it forms a high-selectivity, low-conductance water channel (26). Finally, reconstitution results in a random orientation of the aquaporin in the proteoliposome, which makes it more difficult to study the effects of phosphorylation on channel function (23, 47).

The present study seeks to develop a method for studying aquaporin function that combines the advantages of oocytes with those of reconstitution but avoids many of the disadvantages. First, because the starting material is a cDNA, it is theoretically possible to express any native or mutant aquaporin in the yeast system. Second, because the yeast inserts the protein into the secretory vesicle as it is being made (6), in a process similar to the process that occurs in the native tissue, there is little chance that aquaporin function will be altered during expression. Third, the solutions inside and outside of the vesicles can be modified systematically, permitting detailed definition of channel function. Fourth, the channels are likely to be inserted into the vesicles in a cytoplasmic-side out configuration, permitting studies of the effects of phosphorylation on channel function. Finally, as demonstrated here, the yeast system may serve as a starting point for purification and reconstitution experiments.

The present study demonstrates the utility of this approach for AQP1 and AQP2 in that the vesicles containing the channel exhibited markedly increased water permeability with a low activation energy. In this

Fig. 5. Water flow in secretory vesicles from yeast expressing AQP2 (B) and control yeast (A). Because the vesicles containing AQP2 exhibited an extremely rapid rate of water flow, the early portion of the curve is shown in the inset.

Fig. 6. Activation energy for water flow in secretory vesicles from untransformed yeast (Control) and yeast expressing AQP2 (AQP2). Different graphs are used because rate constants were very different and because temperatures at which AQP2 vesicles could be studied were limited.
respect, these studies agree with those published by Laize et al. (21), in which successful expression of AQP1 in yeast secretory vesicles was demonstrated. In their studies, expression of AQP1 led to a threefold increase in secretory vesicle water permeability. Although this was sufficient to demonstrate functional AQP1 expression, the modest increase in water permeability obtained would not permit a biophysical definition of aquaporin function. With optimization of induction protocols, we have increased water flow by 14- to 40-fold in the case of AQP1 and by ~64-fold in the case of AQP2. These dramatic increases in water permeability will permit biophysical definition of aquaporin function.

Solubilization of the protein extracted from yeast secretory vesicles followed by its reconstitution into proteoliposomes gave results identical to those published previously, establishing the yeast secretory vesicle system as a viable source of aquaporin protein for reconstitution (49, 50). Because AQP1 has been prepared for two-dimensional crystallization studies in proteoliposomes, the yeast expression system opens the way for structural studies of aquaporins that cannot be isolated in quantity directly from their native tissues (45, 46).

AQP2 expression in secretory vesicles also led to dramatic increases in water permeability, again at low activation energy. The vesicles were then used to define the selectivity of this channel for water over other substances. Several members of the aquaporin family, including AQP3, NOD26, and glpF, exhibit high permeabilities to glycerol and urea and appear to exhibit variable permeabilities to water (10, 17, 24, 27, 32). Studies of the selectivity of AQP2 in oocytes have given conflicting results, with most authors reporting little to no solute permeability and one group reporting appreciable permeability to glycerol and urea (1, 12). In the present studies, we were unable to demonstrate any ability of AQP2 to increase the permeability of secretory vesicles to urea, formamide, or glycerol despite a ~60-fold increase in water permeability. Water, formamide, urea, and glycerol represent a range of radii for solutes, with van der Waals volumes of 10.6, 24.8, 32.8, and 51.4 cm³/mol (5, 44), respectively. If the solutes are assumed to have van der Waals volumes that occupy spheres, this suggests that the pore radius is ~1.6 Å but ~2.1 Å. The results of the present studies agree with those published by Laize et al. (21), in which successful expression of AQP1 in yeast secretory vesicles was demonstrated. In their studies, expression of AQP1 led to a threefold increase in secretory vesicle water permeability. Although this was sufficient to demonstrate functional AQP1 expression, the modest increase in water permeability obtained would not permit a biophysical definition of aquaporin function. With optimization of induction protocols, we have increased water flow by 14- to 40-fold in the case of AQP1 and by ~64-fold in the case of AQP2. These dramatic increases in water permeability will permit biophysical definition of aquaporin function.

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study indicate that AQP2, like AQP1, has a narrow pore that is highly selective for water.

In previous studies, we have shown that AQP1 is highly selective for water over protons (49, 50). However, vasopressin stimulation of toad urinary bladder granular cells causes a concurrent increase in water and proton permeabilities (14). Moreover, gramicidin forms single-file water channels that functionally resemble aquaporins and conduct protons rapidly (11). Because AQP2 is functionally similar to the aquaporin found in toad bladder, proton flux across AQP2 could increase proton permeability of the collecting duct whenever vasopressin-mediated water reabsorption occurs (14, 15). Our results provide the most direct evidence to date that AQP2 does not conduct protons. This is physiologically consistent, since vasopressin-stimulated concentration of the urine should not simultaneously require deacidification of the urine via proton leak through collecting duct principal cells. The results also suggest that the water pore through AQP1 and AQP2 has a barrier to the flow of charged species, such as protons (50).

AQP1 and AQP2 appear to function similarly as high-conductance, high-selectivity aquaporins, which likely feature a narrow, water-selective pore (49, 50). By contrast, other aquaporins, such as AQP3 and glpF, which conduct glycerol and urea, exhibit low water permeability in some cases (24, 27) but high water permeability in others (10, 17). Finally, there may be an intermediate class of aquaporins, such as NOD26 and AQP0, which exhibit moderate water conductance and may, under some circumstances, mediate fluxes of solutes (13, 26, 31, 32, 48, and R. Rivers, D. M. Roberts, and M. L. Zeidel; unpublished observations). Further studies of the biophysical properties of these aquaporins using the yeast expression system will permit clarification of the relationship between aquaporin primary structure and function.

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