Transport of Water and Glycerol in Aquaporin 3 Is Gated by H**

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Aquaporins (AQPs)† are a class of membrane proteins that allows osmotic water transport probably via an aqueous pore (1). Some AQPs can transport other solutes as well, AQP3 for example, supports significant fluxes of glycerol (2–7). It has long been known that glycerol transport across the plasma membrane of the red blood cell is mediated by a pore and that the transport mechanism is inhibited at low pH (8, 9). Recently it was established that AQP3 is involved in glycerol transport in the red blood cell (10). This raises the questions of whether glycerol transport in AQP3 is gated by H+ and whether water transport through AQP3 and other AQPs is also sensitive to H+.

We have previously applied a fast and high resolution optical method to determine the transport properties of AQPs expressed in Xenopus oocytes (6). Here we combine this method with tracer measurements in order to study the effects of H+ temperature, and solute structure on transport of water, glycerol, and other straight chain polyols. The study is performed predominantly in AQP3, but also in AQP0, AQP1, AQP2, AQP4, and AQP5.

At present, transport models are restricted to the use of macrophysical concepts such as pore diameter and pore length. This is mainly due to the lack of knowledge about the structure of the putative pore and the nature of the chemical interactions with the permeating molecules (11, 12). Our data for AQP3 suggest a model where the permeation of water and polyols are determined by the formation of hydrogen bonds between the pore and the permeating molecule. From a physiological point of view, it is interesting that both the glycerol and the water transport through the AQP3 exhibited a strong, immediate, and reversible pH dependence. Such short term and direct gating of transport is a novel feature of aquaporins.

MATERIALS AND METHODS
Details of the preparation of mRNA, the preparation and injection of Xenopus oocytes, and the set-up for Lp and σ measurements have been described in detail previously (6, 13, 14). For measurements of the water permeability (Lp) and reflection coefficients (σ) oocytes were placed in a chamber (30 μl) in which solution changes could be accomplished within 5 s (90% complete). The oocytes were stabilized by the insertion of two microelectrodes, which also recorded the membrane potential. The presence of the microelectrodes did not affect the measurements (6, 14). Oocyte volumes were monitored on-line with an accuracy of 0.03% equal to about 0.4 nl, via an inverted microscope connected to a charge coupled device camera. Lp and σ were obtained from the initial rate of volume decrease induced by the addition of 20 mosmol of test solution to the control bathing solution. The σ of a given polyol was obtained from the ratio between the volume changes induced by the polyol and mannitol. The control bathing solution contained in mm: 90 NaCl, 20 mosmol of mannitol, 2 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES or MES, osmolarity 214 mosmol; pH was adjusted according to the type of experiment. Test solutions were obtained by adding 20 mosmol mannitol or one of the following straight chain polyols (mole weights and boiling points (°C), respectively, in parentheses): ethylene glycol, EG (62, 197); 1,2-propanediol, PD (76, 187); glycerol, Gl (92, 193); 1,2-butandiol, B12 (90, 184); 1,3-butandiol, B13 (90, 184); 1,4-butandiol, B14 (90, 230); 2,3-butandiol, B23 (90, 184); 1,5-pentandiol, P15 (104, 242); 2,4-pentandiol, P24 (104, 201); the structures are indicated in Fig. 1B.

The isotonic test solution was obtained by replacing 20 mosmol of mannitol in the control solution by 20 mosmol of glycerol.

The glycerol permeability, PGl, was derived from [14C]glycerol uptake. The oocytes were equilibrated for at least 2.5 min in the given test solution before being transferred for 0.5–10 min to 3 ml of the stirred test solution to which 4 μCi ml−1 [14C]glycerol (Amersham Pharmacia Biotech, code CFB 174, ethanolfree) had been added. To terminate uptake, oocytes were washed twice in ice-cold medium and transferred to scintillation vials, incubated for approximately 1 h at room temperature with 1 ml of 20% SDS before addition of 15 ml of scintillation fluid (Packard Opti-Fluor), and counting in a scintillation counter (Packard Tri-Carb).

The Lp and PGl were analyzed as functions of external pH by fitting to a Boltzmann function: exp[l(pH−pK/ΔH)]/(1+exp[l(pH−pK/ΔH)])

ΔH is a measure of the steepness of the curve around pK and is related to the Hill coefficient n = d log Lp/d log(pH) via n = 1/(2.3 ΔH).

A phenomenological analysis of the volume and solute fluxes and the coupling between them was obtained from irreversible thermodynamics (15), with glycerol as an example,

\[ J_v = -A^* R^* T^* L_{p^*}(ΔC + σ_{p^*} ΔC_{p^*}) \]  
\[ J_{gl} = A^* R^* T^* P_{gl^*}\DeltaC_{gl} + J_v C_{v^*} σ_{v^*}(1 - σ_{v^*}) \]  

where Jv is the volume flow, determined from the initial rate of change
in oocyte volume. \( J_{gl} \) is the flux of glycerol, determined from tracer uptake. \( A \) is the true oocyte surface area: with an average diameter of 1.35-mm, oocytes have an apparent surface area of 5.9 mm\(^2\). Folding of the membrane increases this area by a factor of nine (16) to give the true surface area \( A = 0.53 \text{ cm}^2 \). \( R \) is the gas constant and \( T \) the absolute temperature. \( \Delta C_i \) is the transmembrane concentration difference of impermeable solutes such as mannitol; \( \Delta C_{gl} \) is the difference in glycerol concentration. \( C_{gl} \) is the average concentration of glycerol in the aqueous pore. The coupling between the solute and volume fluxes in the aquaporin can be characterized by \( \sigma \) (Equations 10-56 in Ref. 15),

\[
\sigma_{gl} = 1 - R T P_{gl} (\Delta C_{gl}) f_{sw} \tag{Eq. 3}
\]

where \( \Delta C_{sw} \) is a constant that gives the ratio of the membrane thickness to the volume fraction of water. \( f_{sw} \) is a formal frictional factor that gives the coupling between the solute (glycerol) and water in the pore. Changes in \( \sigma \), which arise from partial molar volume effects, are ignored because they were too small to affect the measured values. It follows that a \( \sigma \) significantly smaller than 1 is evidence for interaction between solute and water and that 1 - \( \sigma_{gl} \) is proportional to the glycerol permeability. \( \sigma_{gl} \) times the friction, \( f_{sw} \). We will refer to this term as the interaction and relate Arrhenius activation energies (\( E_a \)) to this.

The parameters presented have been corrected for the fluxes taking place via the membrane of the native oocytes. All numbers are given as means \( \pm \) S.E., unless otherwise stated the number in parentheses is the number of experiments in at least four oocytes.

**RESULTS**

\( L_p \) and \( \sigma \) at pH 7.4—For AQP3 the \( L_p \) was 6.9 \( \pm \) 0.3 (10\(^{-5} \text{ cm s}^{-1} \text{ osmol liter}^{-1} \)) (29 oocytes). The \( \sigma \) values for the polyols were all smaller than 1, for data see Fig. 1. For AQP1 the \( L_p \) was 6.8 \( \pm \) 0.3 (10\(^{-5} \text{ cm s}^{-1} \text{ osmol liter}^{-1} \)) (8 oocytes). \( \sigma_{gl} \) was 0.94 \( \pm \) 0.01 (35), \( \sigma_{PP} \) was 0.93 \( \pm \) 0.02 (32), and \( \sigma_{EG} \) was 0.95 \( \pm \) 0.01 (36), all significantly lower than 1.

The possibility that the test solutes crosses the membrane via another route than the AQPs and diminishes the driving force during the measurement can be excluded (6). Consider in the present investigation the most permeable test solute, EG. If a flux of EG (via the membrane or in between the membrane and the AQPs) should lead to any significant intracellular accumulation within the test period of 10-20 s, then \( \sigma_{EG} \) would be small for both AQP3 and AQP1. In fact, \( \sigma_{EG} \) for AQP3 was close to zero, for AQP1 it was about one.

**pH Dependence of \( L_p \), \( \sigma \), and Membrane Potential**—The \( L_p \) of AQP3 was immediately affected by shifts in external pH. When the \( pH \) of the bath solution was changed to acidic values, the \( L_p \) began to decrease within 1-3 s (compare Fig. 2, A and B). The inhibition was complete in about 60 s, at which time \( L_p \) equaled that of native oocytes. To ensure steady states at a given \( pH \), oocytes were adapted for at least 2 min before \( L_p \) and \( \sigma \) were recorded. The steady state \( L_p \) had a marked \( pH \) dependence with a \( pK \) of 6.4 \( \pm \) 0.01, a Hill coefficient of 2.7 \( \pm \) 0.3 (\( x^2 = 0.017 \), 119 recordings from nine oocytes), and complete inhibition at \( pH \) values below 5.5 (Fig. 2C). The inhibition was reversible; at the return to external \( pH \) of 7.4, \( L_p \) immediately began to recover (Fig. 2B) and was normalized in about 60 s. In
about one-third of the oocytes, changes in bathing solution pH under isotonic conditions resulted in short lasting (typically 2 s) transient changes in oocyte volume of about 0.1%; we have no explanation of this phenomenon. The $L_p$ values of the other AQPs were insensitive to external pH. The $L_p$ of AQP1 was measured for pH values between 7.4 and 4.5 (Fig. 2C). For AQP0, AQP2, AQP4, and AQP5 there were no significant differences between the $L_p$ values measured at pH 7.4 and 4.5 (data not shown).

For AQP3 the reflection coefficients for glycerol ($\sigma_{Gl}$) and formamide ($\sigma_{form}$) were sensitive to external pH. In steady state $\sigma_{Gl}$ was $0.15 \pm 0.01$ (15) at pH 7.4 and $0.32 \pm 0.02$ (23) at pH 6.4. $\sigma_{form}$ was $0.23 \pm 0.04$ (13) at pH 7.4 and $0.53 \pm 0.03$ (24) at pH 6.4. The changes were reversible.

Acid pH reduced the membrane potential of the oocytes. At pH 7.4 the average potential was $-43 \pm 2$ mV (35 oocytes). When the external pH was lowered the potential began to depolarize within 1 s and became stable after about 60 s. At pH 4.5 the steady state potential was $-18 \pm 2$ mV (nine oocytes); between these values the steady state potential was a linear function of external pH. The membrane potential began to recover within 1 s when external pH was returned to 7.4 and stabilized after about 60 s. The pH effect was independent of which type of aquaporin that was expressed and must be ascribed to reversible modulations by pH of ion channel activity in the oocyte plasma membrane.

Temperature Dependence—For AQP3, $\sigma_{Gl}$ decreased with increasing temperature from $0.24 \pm 0.01$ at 15.6 °C (26) to $0.13 \pm 0.02$ at 22 °C (23) and to $-0.23 \pm 0.03$ at 31 °C (5). In the temperature range 15.6 to 31 °C, the Arrhenius activation energy ($E_a$ in kcal mol$^{-1}$) for (1 - $\sigma_{Gl}$) was $4.8 \pm 0.23$ (54). $E_a$ was higher in the range 31 to 22 °C ($7.2 \pm 0.45$ kcal mol$^{-1}$) than in the range 22 to 15.6 °C ($3.4 \pm 0.6$ kcal mol$^{-1}$). Oocytes only survived about 10 min at 31 °C as judged from the membrane potential. $E_a$ for (1 - $\sigma_{Gl}$) was $4.5 \pm 1.6$ kcal mol$^{-1}$ (four oocytes) and for (1 - $\sigma_{Gl}$) it was $4.8$ kcal mol$^{-1}$ (two oocytes), measurements at 15 and 22 °C.

Glycerol Uptake by AQP3—The uptake rate for glycerol decreased with time, but was practically constant for the first min in the following experiments (Fig. 3A). In hypertonic test solutions, where 20 mM of glycerol was added to the control bathing solution at pH 7.4, the initial rate of uptake ($J_{Gl}$) was $24 \pm 0.15$ (10$^{-9}$ mol min$^{-1}$ oocyte$^{-1}$) (15 oocytes). Given the average surface area of 0.53 cm$^2$ this gives a $P_{Gl}$ of 2.8 ± 0.25 (10$^{-6}$ cm s$^{-1}$) (Fig. 3B). The uptake into native oocytes was measured to be less than 4% of $J_{Gl}$ (Fig. 3A) and has been corrected for in $P_{Gl}$. $P_{Gl}$ was independent of whether it was recorded during an influx or an efflux of water. Oocytes swelled in isotonic test solution where 20 mM glycerol replaced mannitol (6). Under these conditions $P_{Gl}$ was $0.99 \pm 0.06$ (15 oocytes) times the $P_{Gl}$ recorded in the hyperosmolar test solution used above. This shows that solvent drag in the pore is insignificant, i.e. that the second term on the right-hand side of Equation 2 can be disregarded and that the derivation of $P_{Gl}$ by means of hypertonic solutions is valid.

$P_{Gl}$ was reduced at lower temperatures. A comparison of $P_{Gl}$ measured at 23 and 10 °C gave an $E_a$ of $5.6 \pm 0.5$ kcal mol$^{-1}$ (eight oocytes).

$P_{Gl}$ was a function of the pH of the test solutions (Fig. 3B). It was independent of pH values down to about 6.25, decreased steeply for more acid pH values, and was abolished at a pH of about 5.6. The $pK$ was $6.1 \pm 0.04$ and the Hill coefficient $6.2 \pm 1.6$ ($\chi^2 = 0.04$, 90 oocytes).
DISCUSSION

AQP3 has been found to act as a channel for both water and glycerol transport. The fluxes are linear functions of their respective chemical driving forces (3, 5, 6, 17), and the activation energies are low (3, 6). Furthermore, the two fluxes interact in the protein (6). Our data show that the transports are gated by H⁺. In principle, three simultaneously active groups of titratable sites could be responsible for this behavior: one group in which titration led to closure of the channel(s), another that controlled the \( L_p \), and finally one that controlled the \( P_{Gl} \). We will discuss the simplest possibility: that these groups are, at least partially, identical.

Mechanisms and Coupling of Water and Glycerol Fluxes—

The behavior of AQP3 cannot be interpreted in terms of a physical pore. AQP3 remained open to glycerol transport in the pH range 5.8–6.2 while being closed for the smaller water molecule. The data can be interpreted by means of an Eyring energy barrier model (18). On this model, the molecule permeates by a series of jumps, the energy barriers of which are determined by the chemical bonds between the molecule and specific sites in the pathway. For AQP3, the \( E_a \) for \( L_p \) was low, around 5 kcal mol\(^{-1}\), which suggests that the water molecule at neutral pH crosses this aquaporin by forming a succession of single hydrogen bonds. The \( L_p \) exhibited an immediate and reversible dependence on external pH (Fig. 2, A and B), under steady state conditions the \( L_p \) depended in a sigmoidal manner on external pH with a \( pK \) of 6.4 and a Hill coefficient of about 3. This suggests that at least three cooperating titratable sites determine the \( L_p \). In the simplest, but not the only, model these sites are located in the aqueous pathway and determine the energy barriers for water permeation. Titration of the sites would abolish their hydrogen bonding capacity and render them effectively hydrophobic. In analogy to the \( L_p \), the \( P_{Gl} \) had low activation energy and a marked dependence on external pH. But the \( pK \) was lower, 6.1, and the Hill coefficient larger, about 6 (Fig. 3B). This would suggest that glycerol also permeates by forming successive hydrogen bonds, the Hill coefficient indicates at least six.

The difference between \( pK \) values and Hill coefficients raises the question whether it is the same titratable groups that determine the \( L_p \) and \( P_{Gl} \). We suggest it is and that the difference arises from two effects. First, glycerol with its three —OH groups might have to make and break more hydrogen bonds than water in order to cross the aquaporin; this would lead to a higher Hill coefficient. Second, the \( pK \) for glycerol transport could be shifted due to a competitive interaction between H⁺ and glycerol at the sites. Such competition has been described in intact human red blood cells (9) where glycerol transport is mediated by AQP3 (10). In these cells \( pK \) for \( P_{Gl} \) was about 6.0 at external glycerol concentrations of 1 mM and 5.5 at external glycerol concentrations of 2 M. In addition, the Hill coefficients were estimated to be larger than 2 (9). These values are in agreement with those of the present study where glycerol concentrations of 20 mM were employed. It appears that glycerol, when close to the titratable site, to a certain extent displaces water molecules, an effect that would be enhanced by the
confinement of the pore. The resulting lower molar fraction of water near the site would result in a lower local H⁺ concentration and consequently in a decrease of the effective pK. The hypothesis of a pathway shared by water and glycerol in AQP3 is supported by the finding that σGl doubled when external pH was lowered to 6.4. This shows that the pathways for water and glycerol have at least one titratable site in common with a pK around 6.4. Titration reduces the availability of this site, and the interaction between the fluxes is reduced. At sufficiently acid pH values both water and glycerol would be unable to cross the channel. The fact that σ for formamide also doubled at pH 6.4 supports the notion that it is the —OH groups of the solute rather than its backbone that is responsible for interaction.

Our model suggest a mechanism of how another member of the major integral protein family, the glycerol facilitator GlpF, can act as a glycerol channel without letting through water (19). If the pore of GlpF had a titratable site that was protonated at normal pH, it would be hydrophobic and in effect prevent the passage of water. If the site allowed competitive interaction between glycerol and H⁺ as described above, the glycerol molecule would be able to remove the H⁺ and use the site for transport.

The model is not directly applicable to AQP0, AQP1, AQP2, AQP4, and AQP5, since they did not exhibit any pH sensitivity. One possibility is that the sites responsible for Lp in these aquaporins are not accessible to H⁺. AQP1 has a small but significant permeability to glycerol (6, 20, 21), and the σ values for the smaller polyols, EG and PD, were of the same size as that of glycerol. This shows that although these polyols interacts with water in AQP1, some structural incompatibility, not found in AQP3, prevents them from permeating at any larger rate.

The Role of Polyl Composition—In general the σ values for AQP3 increased with the number of —OH groups and number of carbons of the test solute (Fig. 1B). The importance of —OH groups available for hydrogen bonding was particularly clear when σ values of the butanols B12, B13, B14, and B23 were compared. For these polyols the location and intramolecular interactions of the two —OH groups had significant specific effects on σ values. The σ values were larger if the two —OH groups were located next to each other and engaged in intramolecular bonding (σB23 > σB12 > σB13 = σB14). The extent of intramolecular bonding was mirrored by the boiling points, which were lower for B12 and B23 than for B13 and B14. The —OH groups in B12 and B23 were therefore not available for interaction with the sites in the aquaporin to the same degree as the —OH groups of B13 and B14. This would result in smaller fluxes (smaller P) and/or smaller frictions with the water (smaller fsw) and therefore in larger σ values for B23 and B12 (Equation 3). The effects of the locations of the —OH groups on σ were absent for the pentanols. Most likely the longer carbon chain mitigate the strength of intramolecular bonding between —OH groups as witnessed by the small variations between the boiling points among this group.

The picture that emerges is one where the test molecules, viewed as cylinders of different lengths and roughly similar diameters, cross the pore of AQP3 with their axis parallel to the pore. During permeation the —OH groups of the solute form a succession of single hydrogen bonds with the aquaporin as indicated by the low activation energies of around 5 kcal mol⁻¹ observed for JGl 1 – σGl 1 – σB13, and 1 – σB23.

Comparison with Other Studies—The numerical values for the transport parameters derived here and in an earlier paper (6) are compatible with the majority of published data of others (3, 5, 17). Only one report gives a high Er for PGl (5); we have no explanation for this discrepancy. The glycerol transport in AQP3 has been reported to be independent of external pH in the range 6–7 (22), while we found PGl to be significantly smaller at pH 6.0 (Fig. 3B). The same investigators (22) also reported no water permeability of the AQP3, which is in contrast to all other reports (3, 5, 6, 17) and the present study.

The σGl determined by us in a previous study 0.24 (6) was higher than the one determined here, 0.15. The oocytes in the present study had more negative membrane potentials, on average ~43 mV compared with the ~25 mV in the previous study. As σGl was found to increase with acidity, we suggest that the oocytes used previously, being more stressed, might have had a more acid intracellular pH.

Relation between Primary Structure and Permeation in AQP3—The ability of AQP3 to transport both water and larger solutes is shared by AQP7 (24, 25) and AQP9 (17). Functionally, this places these three aquaporins in between those members of the major integral protein family that transport predominantly water (i.e. AQP0, AQP1, AQP2, AQP4, AQP5) and those that are impermeable to water, i.e. the glycerol facilitator GlpF (19). In view of the unique dependence of AQP3 on pH, titratable residues common for AQP3 and the water transporting aquaporins may not be relevant to explain the transport properties, while homology with the glycerol transporting aquaporins may be more important. If single residues are focused upon, an obvious guess for the titratable sites would be histidines, which qua their imidazole ring have pK values of 6.0–7.0 when incorporated into proteins. Other candidates with hydrophilic side groups are aspartate and glutamate residues, which may have pK values as high as 7 in proteins. All three amino acid residues are known to participate in hydrogen bonding (23).

Interestingly, specific structural changes in aquaporins have been shown recently to cause shifts from water to glycerol permeation (26, 27). It should be investigated whether this phenomena and the one described by us have a common basis. The rapid and reversible effects of H⁺ observed by us, however, do not per se implicate structural or major conformational changes.

Physiological Relevance—AQP3 has been localized in several mammalian tissues: eye, kidney, stomach, spleen, intestine, and erythrocytes (3, 28–30). For a recent review, see Ref. 7. The ability of AQP3 to transport both water and larger solutes is shared by AQP7 (24, 25) and AQP9 (17). Functionally, this places these three aquaporins in between those members of the major integral protein family that transport predominantly water (i.e. AQP0, AQP1, AQP2, AQP4, AQP5) and those that are impermeable to water, i.e. the glycerol facilitator GlpF (19). In view of the unique dependence of AQP3 on pH, titratable residues common for AQP3 and the water transporting aquaporins may not be relevant to explain the transport properties, while homology with the glycerol transporting aquaporins may be more important. If single residues are focused upon, an obvious guess for the titratable sites would be histidines, which qua their imidazole ring have pK values of 6.0–7.0 when incorporated into proteins. Other candidates with hydrophilic side groups are aspartate and glutamate residues, which may have pK values as high as 7 in proteins. All three amino acid residues are known to participate in hydrogen bonding (23).

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