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Glycerol permeability of mutant aquaporin 1 and other AQP-MIP proteins: inhibition studies

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Abstract In a recent work, we showed that the aquaporins 1 (AQP1) are permeable to certain small solutes such as glycerol. Here, we have further investigated the permeation pathway of glycerol through human AQP1 (hAQP1) by the use of mutants (C189S, H180A, H209A) and inhibitors such as P-chloromercuribenzenzene sulphonate (pCMBS), CuSO₄ or phloretin, in comparison with other AQP-MIP (where MIP denotes major intrinsic protein) proteins: hAQP2, plant water channel γTIP and bacterial glycerol permease facilitator, GlpF. Glycerol movements were measured in Xenopus laevis oocytes. Apparent glycerol permeability coefficients ($P_{gly}^\text{a}$) were calculated from the rates of oocyte swelling upon exposure to an isoosmotic medium containing an inwardly directed gradient of glycerol and from $[^3\text{H}]$glycerol uptake measurements. Similar $P_{gly}^\text{a}$ values were obtained for hAQP1 and hAQP2, 6 to 8 times greater than control indicating that hAQP2 also transports glycerol. $P_{gly}^\text{a}$ of hAQP2-injected oocytes was pCMBS and CuSO₄ sensitive. In contrast, the $P_{gly}^\text{a}$ value of γTIP was close to that of control, indicating that γTIP does not transport glycerol. The hAQP1-C189S, -H180A and -H209A mutants gave $P_{gly}^\text{a}$ values similar to those obtained for wild hAQP1, indicating that these mutations did not affect glycerol movements. However, the H209A mutant has an osmotic water permeability coefficient ($P_o$) value decreased by 50%. The inhibitory effect of pCMBS on $P_{gly}^\text{a}$ was maintained for the 2 His mutants and, more interestingly, was also conserved for the C189S mutant. CuSO₄ significantly inhibited $P_{gly}^\text{a}$ of oocytes expressing hAQP1, hAQP1-C189S, -H180A, and -H209A mutants and had no effect on $P_{gly}^\text{a}$ of GlpF-injected oocytes. Phloretin was shown to inhibit by around 80% the glycerol fluxes of wild and mutant hAQP1, hAQP2 and to fully inhibit glycerol uptake in GlpF-injected oocytes.

Key words Aquaporins · AQP-MIP proteins · Mutants · Glycerol permeability · Xenopus oocytes · pCMBS · Copper · Phloretin

Introduction

Aquaporins (AQPs) constitute a family of intrinsic membrane proteins found in the plasma membrane of numerous water-transporting tissues (for review, see [3, 24]). Their known function is to conduct water in the direction of an osmotic gradient. The first AQP (CHIP28 or AQP1) was discovered by Agre and his colleagues in human erythrocytes [6]. The gene was cloned and sequenced [20] and the water-channel function of AQP1 was demonstrated by expression studies in Xenopus oocytes [21] and by functional reconstitution in proteoliposomes [26]. AQP1-CHIP was subsequently found to be expressed in segments of the mammalian nephron constitutively permeable to water, i.e. the proximal tubule and the descending limb of Henle's loop [28] and in the frog urinary bladder [1]. In contrast, AQP2 or WCH-CD [8] represents a vasopressin-regulated water channel addressed to the apical membrane of collecting duct principal cells after hormonal stimulation [19].

Until the discovery of AQP3 [11], which was found to be permeable to urea and glycerol in addition to water, it was considered that AQPs were exclusively...
selective for water [27]. In our previous work [2], we showed that human, rat and frog AQP1 facilitate the permeation of small non-electrolytes such as glycerol but exclude urea. The apparent glycerol permeability \( (P_g) \) was strongly inhibited by \( P \)-chloromercuribenzenesulphonate (\( p \)-CMBS) or \( HgCl_2 \) and, to a lesser extent, by a specific inhibitor of glycerol fluxes in red blood cells, \( Cu^{2+} \) [4]. Inhibition by \( Cu^{2+} \) was reversed after incubation with a copper-binding peptide, Gly-Gly-His [12]. These observations and glycerol reflection coefficient calculations led us to conclude that AQP1 constituted a facilitated pathway for glycerol and certain other small solutes.

In the present work, we further investigate the permeation pathway of glycerol through human AQP1 (hAQP1) and human AQP2 (hAQP2) [5] in comparison with the bacterial glycerol permease facilitator, GlpF [17], and the plant water channel, \( \gamma TIP \) [16], by the use of mutants and inhibitors such as \( p \)-CMBS, \( Cu^{2+} \) or phloretin. We were particularly interested in elucidating the inhibitory action of \( Cu^{2+} \) on glycerol transport. Indeed, the interactions of \( Cu^{2+} \) ions with specific residues of CHIP28 are of importance in the understanding of the glycerol permeation pathway. Due to the very high affinity of \( Cu^{2+} \) for His [12], we decided to perform two independent point mutations at histidine residues at position 180 (\( His^{180} \)) and 209 (\( His^{209} \)) on hAQP1. These residues, highly conserved in the AQPs, are supposed to be located on the external side of the membrane, i.e. easily accessible. Ala was chosen to substitute for His, because it is assumed to have no important effects on the protein conformation. Inhibitory studies were carried out with oocytes injected by the complementary RNA (cRNA) of hAQP1 and its C189S [22,29], H180A and H209A mutants, by hAQP2-, by GlpF- and by \( \gamma TIP \)-cRNA.

Materials and methods

Site-directed mutagenesis, in vitro cRNA synthesis and oocyte preparation

Expression vectors and cDNAs

DNA manipulations were performed according to standard practice [23]. Restriction and modification enzymes were from New England Biolabs, except for \( Tsiq \) polymerase (Beckmann), and T4 DNA ligase (Boehringer).

The cDNAs coding for hAQP1 and hAQP1-C189S were kindly provided by Dr. G. Preston. The \( E. coli \) GlpF cDNA was a gift from Dr. C. Maurel. These three cDNAs were cloned into the EcoRI site of the expression plasmid pSP64T, a Bluescript-derived vector containing the 3' and 5' untranslated sequences from the \( Xenopus laevis \) \( \beta \)-globin gene, a gift of Dr. Krieg. The cDNA coding for hAQP2 was cloned into the EcoRV site of the pt7TS plasmid, a pGEM4-Z-derived vector containing the same \( Xenopus \) sequences as pSP64T.

Directed mutagenesis

The strategy for directed mutagenesis using polymerase chain reaction (PCR) is depicted in Fig. 1, and has been used to mutate the \( His^{180} \) and \( His^{209} \) of hAQP1 to Ala. The PCRs have been performed directly on the hAQP1 expression vector (pCH1Pev). For each mutation, two mutated oligonucleotides have been used, one in the sense orientation (M1 and M2), and one in the antisense orientation (M1 and M2):

- **M1\(_{180} \)**: 5'-GCC CTT GGA GCC CTC CTG GCT ATT G-3' (Leu Gly Ala Leu)
- **M2\(_{180} \)**: 3'-GA CAT CGG GAA CCT CGG GAG GAC CG-5'

- **M1\(_{209} \)**: 5'-GC AAC GCC TGG ATT TTC TGG GTG G-3' (Ser Asn Ala Trp Ile)
- **M2\(_{209} \)**: 3'-G AAG TCG TTT GGG GAC AAT C-5'

Note that each M1 is complementary to the corresponding M2. The sequence changes leading to the His-Ala transition are underlined (\( His^{180} \): CAC to GCC at position 538; \( His^{209} \): CAC to GCC at position 625). In PCR1 (96° C, 30s; 58° C, 30s; 72° C, 1 min; 30 cycles), M1 has been used with a wild-type sense oligonucleotide (denoted A) located in the 3' untranslated region from the \( Xenopus \) \( \beta \)-globin gene (5'-ACGAATGGCTAGCCATTTTGGGGGACAACATT-3'). In PCR2 (same conditions as PCR1), M2 has been used with a wild-type sense oligonucleotide (denoted S) located between bases 315 and 339 of hAQP1 (5'-CATCGTCGCCACCGCCATCTCTTCA-3'). The products of PCR1 and PCR2 were then mixed and PCR3 (94° C, 30s; 58° C, 30s; 72° C, 1 min; 20 cycles) was performed in the presence of oligonucleotides S and A. In this reaction, two separate events occur, depicted in Fig. 1 as PCR3A (which could be performed without S and A) and PCR3B, leading to the synthesis of the desired mutated PCR product (bottom of Fig. 1).

![Fig. 1 Strategy for directed mutagenesis using polymerase chain reaction (PCR)](image-url)
and multiple other DNA fragments. This product is then purified according to its size (638 bp), digested by PpuMI and BstEII, and the mutated 396-bp PpuMI-BstEII fragment is inserted in pCH1Pev in replacement of the wild-type PpuMI-BstEII fragment. Both mutations were checked by fully sequencing this PpuMI-BstEII region.

Capped cRNA transcripts were synthesized in vitro by using an mCAP mRNA capping kit (Stratagene, La Jolla, Calif., USA). Sense hAQP2 cRNA was synthesized with T7 RNA polymerase and SalI-digested expression vector, and sense GlpF, hAQP1 and hAQPl mutants cRNA were synthesized with T3 RNA polymerase and XbaI-digested expression vector. yTIP cRNA was provided by Dr. C. Maurel.

Stage V and VI oocytes from female X. laevis were injected with either 50 nl water or 10 ng cRNA in 50 nl water. Oocytes were then incubated at 18°C in Barth’s buffer [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid (HEPES)-NaOH pH 7.4, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4] and transport assays were performed 2 days after injection.

Osmotic water permeability measurements

Osmotic water movements were measured at 22°C by using a video microscopy technique to follow the swelling of oocytes suspended in fivefold diluted Barth’s buffer. The initial change in relative oocyte volume with time (d(V/V0)/dt), the oocyte surface area (S), and the molar volume of water (Vw = 18 cm3/mol), were used to calculate the osmotic water permeability coefficient (P0), using the equation:

\[
P_0 = \frac{V_w}{S} \frac{d(V/V_0)}{dt} \frac{(S/V_0)}{(S/V_0)} \]

In a first series of experiments, control and hAQPl- and hAQP2-injected oocytes were preincubated for 30 min in the presence of 0.1 mM diethylpyrocarbonate (DEPC), a His reagent. In other experiments, oocytes were preincubated for 1 h in the presence of 2 mM pCMBS, or for 15 min with 0.05 mM CuSO4, or for 15 min with 0.35 mM phloretin. Some oocytes were preincubated for 30 min in the presence of 5 mM α-mercaptoethanol (ME) to check the reversibility of the inhibition by pCMBS.

“Osmotic” glycerol transport assay

Control and cRNA-injected oocytes were transferred at 22°C from Barth’s buffer (200 mosmol/l) to an isotonic solution containing 160 mM glycerol, complemented with fivefold diluted Barth’s to adjust the osmolarity to 200 mosmol/l. The increase in oocyte volume corresponds to the water influx accompanying the solute uptake. As described above, the initial (d(V/V0)/dt) was calculated and used to determine an apparent glycerol permeability coefficient (Pgly) using the following simplified equation [25]: \( P_{\text{gly}} = \frac{d(V/V_0)}{dt} (S/V_0) \). The same inhibitors were used under the conditions described in the above paragraph; the reversibility of the inhibition by Cu2+ was checked after a 15-min incubation in the presence of 1 mM Gly-Gly-His, a copper-binding peptide.

Radioactive glycerol uptake assay

Tritiated glycerol transport was assayed at 18°C in a solution containing 160 mM [3H]glycerol (final activity of 5.55 MBq/ml) and complemented with fivefold diluted Barth’s solution to adjust the osmolarity to 200 mosmol/l. Individual oocytes were bathed in 200 µl incubation medium for 20 min and were rinsed 3 times with ice-cold Barth’s solution. Oocytes were lysed overnight in 10% sodium dodecyl sulphate (SDS) at room temperature or for 5 min in 2% SDS at 100°C. Radioactivity was measured by liquid scintillation.

Results and discussion

Effects of the inhibitors on \( P_f \)

The \( P_f \) values measured at 22°C in oocytes injected with water, hAQPl, hAQPl-C189S, -H180A and -H209A mutants, hAQP2 and GlpF cRNA are summarized in Table 1. hAQPl, hAQP2 and GlpF cRNA are introduced into oocytes in a common injection buffer containing 160 mM glycerol, complemented with fivefold diluted Barth’s to adjust the osmolarity to 200 mosmol/l. The initial change in relative oocyte volume with time (d(V/V0)/dt), the oocyte surface area (S), and the molar volume of water (Vw = 18 cm3/mol), were used to calculate the osmotic water permeability coefficient (P0), using the equation:

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Results and discussion

Effects of the inhibitors on \( P_f \)

The \( P_f \) values measured at 22°C in oocytes injected with water, hAQPl, hAQPl-C189S, -H180A and -H209A mutants, hAQP2 and GlpF cRNA are summarized in Table 1. hAQPl, hAQP2 and GlpF-H209A cRNA-injected oocytes had a typical high (close to 10-2 cm/s) and mercurial-sensitive osmotic water permeability. In contrast, the \( P_f \) value of the hAQPl-H209A mutant was significantly reduced by 60.7%, suggesting the His259 residue plays a role in the water permeation pathway. Both His mutants conserve their full sensitivity to 2 mM pCMBS and, as expected, the hAQPl-C189S mutant lost its sensitivity towards this inhibitor. In terms of \( P_f \) values, it is interesting to note that the water channel in diluted Barth’s buffer. Others were incubated for 1 h in Barth’s buffer containing 2 mM J-chloromercuribenzenesulphonate (pCMBS) or for 15 min in Barth’s buffer containing 0.05 mM CuSO4 prior to the measurements in diluted Barth’s buffer. Data are expressed as means ± SD of the indicated number of oocytes in parentheses.
AQP3, according to Ishibashi et al. [11] and Echevarria et al. [7], as well as a plant AQP, γTIP [16], are sensitive to mercurials even in the absence of the Cys(189) residue in their sequence, whereas the water channel AQP4 [9, 13], in which the Cys(189) is also absent, is mercurial insensitive. This suggests that sensitivity to mercurials is a complex phenomenon. In all cases, the reducing agent ME (5 mM) reversed the inhibitory effect of pCMBS (not shown). GlpF, which is not a water channel, had a P_f value close to that of control oocytes.

P_f values were absolutely not affected in the presence of 0.05 mM CuSO_4 (Table 1) and 0.35 mM phloretin (not illustrated). In contrast, DEPC, known to specifically carbethoxylate the histidyl residues of proteins without affecting any other amino-acid residues [18], completely inhibits P_f of hAQPl- and hAQPl2-injected oocytes and was without effect on P_f of water-injected oocytes (not shown).

Effects of DEPC on P_gly

DEPC (0.1 mM) was also shown to completely inhibit P_gly of hAQPl1- and hAQPl2-injected oocytes. The P_gly of water-injected oocytes [(1.12 ± 0.21) × 10^{-6} cm/s, n = 96] was not affected, whereas, in the presence of DEPC, the P_gly of hAQPl1-injected oocytes [(8.91 ± 0.74) × 10^{-6} cm/s, n = 43] decreased to (1.25 ± 0.31) × 10^{-6} cm/s, n = 10, and P_gly of hAQPl2-injected oocytes [(9.11 ± 0.42) × 10^{-6} cm/s, n = 26] decreased to (1.52 ± 0.28) × 10^{-6} cm/s, n = 10. Under these conditions, it was impossible to conclude to a specific chemical interaction of DEPC with histidyl residues of hAQPl1 and hAQPl2, since both P_f and P_gly were totally inhibited. This is why we performed the site-directed mutagenesis on the histidyl residues of hAQPl supposed to be the most accessible to Cu^{2+}, the inhibitory action of which seemed more specific (see below).

Effects of pCMBS on P_gly

All the hAQPl mutants (C189S, H180A and H209A) raised oocyte P_gly to values similar to those of the wild hAQPl (Fig. 2), as we observed previously [2]. This indicates that these point mutations did not alter the glycerol fluxes. These results were confirmed by [3H]-glycerol uptake experiments: in water-injected oocytes, P_gly was 0.35 ± 0.048 nmol·min^{-1}·oocyte^{-1} (n = 48) versus 2.46 ± 0.14 (n = 48), 2.26 ± 0.26 (n = 8), 2.03 ± 0.15 (n = 8) and 2.41 ± 0.23 (n = 8) in oocytes injected with hAQPl, hAQPl1-C189S, -H180A and -H209A, respectively.

The inhibitory effect of 2 mM pCMBS on P_gly, already observed in our previous work in hAQPl cRNA-injected oocytes [2], was conserved for the two hAQPl His mutants (Fig. 2) and, more interestingly, was also conserved for the hAQPl1-C189S mutant (Fig. 2A). Since the osmotic water influx was not altered in the latter case, one could assume that glycerol entry would be maintained. This indicates that the glycerol entry is actually the rate-limiting factor in the "osmotic" glycerol uptake. This result also suggests that the Cys residue implicated in mercuric inhibition of P_gly is distinct from that implicated in mercuric inhibition of P_f.

Another important result is that hAQPl2 facilitates glycerol entry in hAQPl2-injected oocytes (Fig. 2), as does hAQPl1, and this was also confirmed.
by the [3H]glycerol uptake: \( P_{gy} \) of control = 0.35 ± 0.048 nmol \( \cdot \) min\(^{-1} \) \cdot oocyte\(^{-1} \) (\( n = 48 \)) and \( P_{gy} \) of hAQP2 = 1.98 ± 0.18 nmol \( \cdot \) min\(^{-1} \) \cdot oocyte\(^{-1} \) (\( n = 18 \)). The inhibition by \( \mu \)CMBS of \( P_{gy} \) and the reversibility of this inhibition by ME were also observed for hAQP2-injected oocytes. This indicates that the difference between these two AQPs cannot be explained in terms of selectivity, but rather in terms of regulatory pathway and tissue localization.

The \( P_{gy} \) of GlpF-injected oocytes was strongly inhibited by 2 mM \( \mu \)CMBS (Fig. 2), as already described by Maurel et al. [17] in [\(^{14}\)C]glycerol uptake experiments. GlpF does not possess the CyS\(_{189}\) residue and this confirms the functional importance of other Cys residues in this protein, as suggested by the authors [17]. Similarly, the glycerol uptake through rat AQP3 was also shown by Ishibashi et al. [11] to be fully inhibited by 1 mM HgCl\(_2\), even in the absence of the CyS\(_{189}\) residue. This result contrasts with those obtained by Ma et al. [15] with GLIP (Glycerol Intrinsic Protein).

Interestingly, as Maurel et al. [16] observed in [\(^{14}\)C]-glycerol uptake measurements and as we confirmed in “osmotic” glycerol assays in oocytes, the plant water channel \( \gamma \)TIP does not transport glycerol \( [P_{gy}\) of \( \gamma \)TIP was \( (1.61 \pm 0.19) \times 10^{-6} \) cm/s (\( n = 5 \)) versus \( (1.4 \pm 0.34) \times 10^{-6} \) cm/s (\( n = 5 \)) in control oocytes].

**Effects of CuSO\(_4\) on \( P_{gy} \)**

This series of experiments was essentially carried out to determine whether the His residues can be involved in the inhibition of glycerol facilitated transport through hAQP1 by Cu\(^{2+}\).

As shown in Fig. 3, the augmented glycerol uptake through hAQP1-H180A and -H209A mutants remained inhibited by 0.05 mM CuSO\(_4\) in the same proportions as the wild hAQP1; this inhibition was reversed after an incubation with the copper-binding peptide Gly-Gly-His. This suggests that the mechanism of action of the divalent Cu\(^{2+}\) is probably more complex than the interaction of Hg\(^{2+}\) with the \(-\)SH group of CyS\(_{189}\) residue and that the replacement of single His residues is insufficient to prevent the inhibitory action of Cu\(^{2+}\). A probable explanation is that Cu\(^{2+}\) strongly binds other His or Cys residues of the protein, which would explain the conservation of the inhibitory effect in the His mutants. If this is the case, it may not only be the CyS\(_{189}\) residue since the inhibition of \( P_{gy} \) by Cu\(^{2+}\) is maintained in the C189S mutant (Fig. 3B).

An inhibitory effect of Cu\(^{2+}\) on glycerol uptake was also observed in hAQP2-injected oocytes that was completely reversed by Gly-Gly-His (Fig. 3B).

In contrast with hAQP1 and hAQP2, \( P_{gy} \) of GlpF-injected oocytes was not inhibited by Cu\(^{2+}\) (Fig. 3B).

This suggests that the permeation pathway of glycerol through GlpF is different from that through the AQPs. Indeed, GlpF is not a water channel and only possesses some 25% amino-acid identity with AQP1 and AQP2 and approximately 40% amino-acid identity with AQP3. Moreover, GlpF transports some polyols larger than glycerol such as tetritol, pentitols or hexitols [10], in contrast with AQP1 [2]. Urca also crosses the GlpF channel [10] but not the AQP1 channel. Moreover, the amounts of transported glycerol in GlpF-injected oocytes were larger than in AQP-injected oocytes (Fig. 3). This suggests that inhibition by Cu\(^{2+}\) of glycerol flux through AQP1 and AQP2 is rather specific.
Effects of phloretin on $P_{\text{gly}}$

Phloretin (0.35 mM) was shown to be an inhibitor of glycerol fluxes in all cases. The percentage inhibitions were 70.1, 80.2, 76, 86 and 83.1% for hAQPI, hAQPI-C189S, -H180A, -H209A and hAQPO2, respectively. 100% inhibition of glycerol entry by phloretin was observed in GlpF-injected oocytes. It has been reported that 0.25 mM phloretin exerts an inhibitory effect on [3H]glycerol uptake by rat AQP3 (GLIP).

In conclusion, our study brings some interesting comparative aspects of glycerol and water permeation pathways through hAQPO1, hAQPO2, the bacterial GlpF and the plant $\gamma$TIP by the use of mutants (hAQPO1-C189S, -H180A, -H209A) and inhibitors ($\mu$CMBS, Cu$^{2+}$ and phloretin). hAQPO2 was shown to transport glycerol as does hAQPO1. GlpF, which is not a water channel, transports glycerol, but the mechanism of glycerol uptake is different from that occurring in hAQPO1 and hAQPO2. Experiments carried out with another water channel, $\gamma$TIP, which does not transport glycerol, demonstrate that certain AQPs are not permeable to glycerol. $\mu$CMBS-induced inhibition of glycerol transport in hAQPO1-C189S-injected oocytes indicates that the Cys residues involved in mercureic inhibition of $P_{\text{gly}}$ and $P_{\text{f}}$ are distinct. Site-directed mutagenesis at two His residues revealed an important role of the hAQPO1 His280 in water transport, since $P_{\text{f}}$ was decreased by a factor of 2. This confirms that loop E of CHIP28 is particularly implicated in the formation of the water channel, as suggested by Jung et al. [14]. However, the mechanism of the inhibitory action of Cu$^{2+}$ on glycerol transport was not elucidated. Consequently, other mutants have to be designed to understand the effect of Cu$^{2+}$, and to elucidate the structure of the glycerol-permeable channel.

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References


