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ORIGINAL ARTICLE

Laurence Abrami · Véronique Berthouaud
 Peter M.T. Deen · Germain Rousselet
 Frédérique Tacnet · Pierre Ripoche

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*-chloromercuribenzenesulphonate (*p*CMBS), CuSO₄ or phloretin, in comparison with other AQP-MIP (where MIP denotes major intrinsic protein) proteins: hAQP2, plant water channel γ TIP and bacterial glycerol permease facilitator, GlpF. Glycerol movements were measured in *Xenopus laevis* oocytes. Apparent glycerol permeability coefficients (P'_{gly}) were calculated from the rates of oocyte swelling upon exposure to an isoosmotic medium containing an inwardly directed gradient of glycerol and from [³H]glycerol uptake measurements. Similar P'_{gly} values were obtained for hAQP1 and hAQP2, 6 to 8 times greater than control indicating that hAQP2 also transports glycerol. P'_{gly} of hAQP2-injected oocytes was *p*CMBS and CuSO₄ sensitive. In contrast, the P'_{gly} value of γ TIP was close to that of control, indicating that γ TIP does not transport glycerol. The hAQP1-C189S, -H180A and -H209A mutants gave P'_{gly} 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_f) value decreased by 50%. The inhibitory effect of *p*CMBS on P'_{gly} was maintained for the 2 His

mutants and, more interestingly, was also conserved for the C189S mutant. CuSO₄ significantly inhibited P'_{gly} of oocytes expressing hAQP1, hAQP1-C189S, -H180A, and -H209A mutants and had no effect on P'_{gly} 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* · *p*CMBS · 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

L. Abrami · V. Berthouaud · G. Rousselet · F. Tacnet
 P. Ripoche (✉)
 Département de Biologie Cellulaire et Moléculaire,
 Service de Biologie Cellulaire, CEA Saclay,
 F-91191 Gif sur Yvette Cedex, France

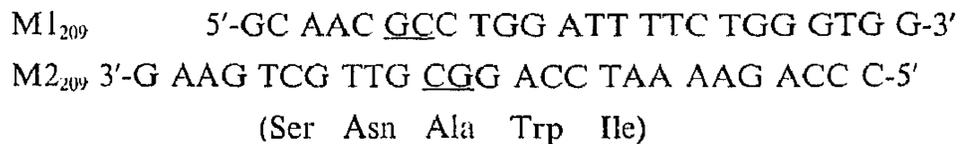
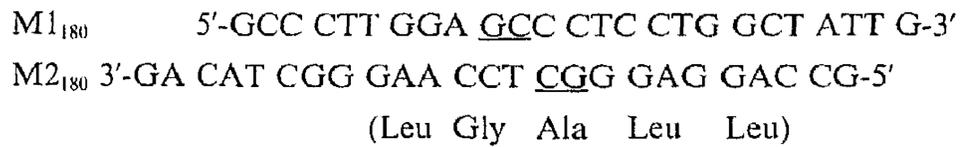
P.M.T. Deen
 Department of Cell Physiology, University of Nijmegen,
 Adelbertusplein 1, PO Box 9101, Nijmegen, The Netherlands

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'_{gly}) 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, γ 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 (His₁₈₀) and 209 (His₂₀₉) 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 γ TIP-cRNA.

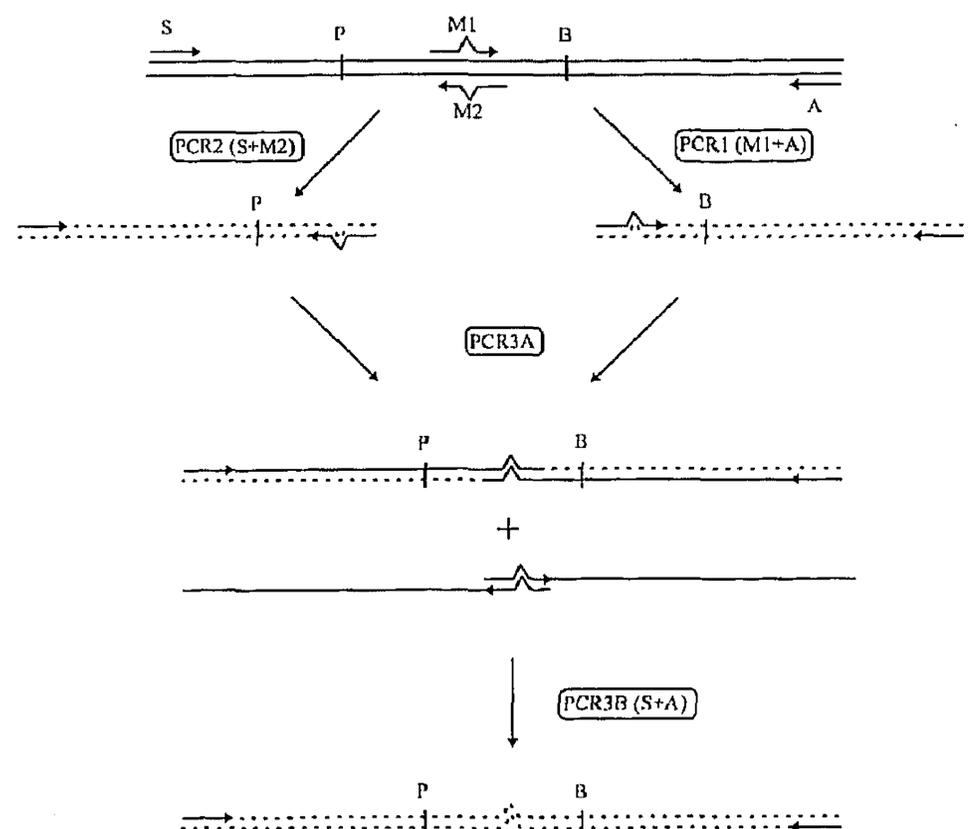
Directed mutagenesis

The strategy for directed mutagenesis using polymerase chain reaction (PCR) is depicted in Fig. 1, and has been used to mutate the His₁₈₀ and His₂₀₉ of hAQP1 to Ala. The PCRs have been performed directly on the hAQP1 expression vector (pCHIPev). For each mutagenesis, two mutated oligonucleotides have been used, one in the sense orientation (M1₁₈₀ and M1₂₀₉), and one in the antisense orientation (M2₁₈₀ and M2₂₀₉):



Note that each M1 is complementary to the corresponding M2. The sequence changes leading to the His-Ala transition are underlined (His₁₈₀: CAC to GCC at position 538; His₂₀₉: 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 antisense oligonucleotide (denoted A) located in the 3' untranslated region from the *Xenopus* β -globin gene (5'-ACGAATGGCTACATTTGGGGGACAACATT-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'-CATCGTCGCCACCGCCATCCTCTCA-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). A complete description of the mutagenesis procedure is given in Materials and methods. For each PCR, newly synthesized DNA fragments are indicated in *dashed lines*. Oligonucleotides are drawn as *arrows* with the *arrowhead* on the 3' side (not to scale). (*P* *Ppu*MI digestion site, *B* *Bst*EII digestion site). Note that PCR3A and PCR3B are performed in the same PCR experiment



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 *Taq* polymerase (Beckmann), and T4 DNA ligase (Boehringer).

The cDNAs coding for hAQP1 and hAQP1-C189S were kindly provided by Dr. G. Preston. The *Escherichia coli* GlpF cDNA was a gift from Dr. C. Maurel. These three cDNAs were cloned into the *Bgl*II site of the expression plasmid pSP64T, a Bluescript-derived vector containing the 3' and 5' untranslated sequences from the *Xenopus laevis* β -globin gene, a gift of Dr. Krieg. The cDNA coding for hAQP2 was cloned into the *Eco*RV site of the pT7TS plasmid, a pGEM4-Z-derived vector containing the same *Xenopus* sequences as pSP64T.

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 pCHIEV in replacement of the wild-type *PpuMI*-*BstEII* fragment. Both mutations were checked by fully sequencing this *PpuMI*-*BstEII* region.

Capped RNA 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 hAQP1 mutants cRNA were synthesized with T3 RNA polymerase and *XbaI*-digested expression vector. γ TIP 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-ethanesulphonic 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 $[d(V/V_0)/dt]$, the oocyte surface area (S), and the molar volume of water ($V_w = 18 \text{ cm}^3/\text{mol}$), were used to calculate the osmotic water permeability coefficient (P_f), using the equation:

$$P_f = V_0[d(V/V_0)/dt]/[S \cdot V_w(\text{Osm}_{\text{in}} - \text{Osm}_{\text{out}})] \text{ with } \text{Osm}_{\text{in}} = 200 \text{ and } \text{Osm}_{\text{out}} = 40 \text{ mosmol/l.}$$

In a first series of experiments, control and hAQP1- 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 *p*CMBS, or for 15 min with 0.05 mM CuSO₄, or for 15 min with 0.35 mM phloretin. Some oocytes were preincubated for 15 min in the presence of 5 mM β -mercaptoethanol (ME) to check the reversibility of the inhibition by *p*CMBS.

"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 external solution to 200 mosmol/l. The increase in oocyte volume corresponds to the water influx accompanying the solute uptake. As described above, the initial $[d(V/V_0)/dt]$ was calculated and used to determine an apparent glycerol permeability coefficient (P'_{gly}) using the following simplified equation [25]: $P'_{\text{gly}} = [d(V/V_0)/dt]/(S/V_0)$. 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 μ l 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, hAQP1, hAQP1-C189S, -H180A and -H209A mutants, hAQP2 and GlpF cRNA are summarized in Table 1. hAQP1, hAQP2 and hAQP1-H180A 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 hAQP1-H209A mutant was significantly reduced by 60.7%, suggesting the His₂₀₉ residue plays a role in the water permeation pathway. Both His mutants conserve their full sensitivity to 2 mM *p*CMBS and, as expected, the hAQP1-C189S mutant lost its sensitivity towards this inhibitor. In terms of P_f values, it is interesting to note that the water channel

Table 1 Inhibition of osmotic water permeability. Osmotic water permeability coefficients (P_f , $\text{cm/s} \times 10^{-3}$) were measured at 22°C in control water-injected oocytes and in oocytes expressing human aquaporin 1 (hAQP1), the mutants hAQP1-C189S, -H180A and -H209A, hAQP2 and the bacterial glycerol permease facilitator GlpF, 2 days after injection. Non-treated oocytes were kept in standard Barth's buffer prior to the measurement of osmotic swelling

in diluted Barth's buffer. Others were incubated for 1 h in Barth's buffer containing 2 mM *p*-chloromercuribenzenesulphonate (*p*CMBS) 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 \pm SD of the indicated number of oocytes in parentheses

cRNA injected	Oocyte treatment		
	None	<i>p</i> CMBS (2 mM)	CuSO ₄ (50 μ M)
Water	1.21 \pm 0.15, (30)	1.17 \pm 0.19, (30)	1.19 \pm 0.22, (11)
hAQP1	12.43 \pm 1.95, (30)	3.33 \pm 0.82, (15)	11.24 \pm 2.33, (15)
hAQP1 C189S	13.24 \pm 2.50, (22)	13.52 \pm 2.54, (15)	12.75 \pm 3.41, (5)
hAQP1 H209A	4.75 \pm 0.55, (24)	1.34 \pm 0.25, (5)	4.51 \pm 0.21, (5)
hAQP1 H180A	12.43 \pm 2.92, (15)	3.11 \pm 0.31, (5)	13.62 \pm 2.51, (5)
hAQP2	12.04 \pm 1.82, (24)	3.95 \pm 0.44, (12)	13.02 \pm 5.20, (10)
GlpF	2.12 \pm 0.22, (20)	3.19 \pm 0.22, (10)	2.05 \pm 0.24, (10)

AQP3, according to Ishibashi et al. [11] and Echevarria et al. [7], as well as a plant AQP, γ TIP [16], are sensitive to mercurials even in the absence of the Cys₁₈₉ residue in their sequence, whereas the water channel AQP4 [9, 13], in which the Cys₁₈₉ 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 *p*CMBS (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 CuSO₄ (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 hAQP1- and hAQP2-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 hAQP1- and hAQP2-injected oocytes. The P'_{gly} of water-injected oocytes [$(1.12 \pm 0.21) \times 10^{-6}$ cm/s, $n = 96$] was not affected, whereas, in the presence of DEPC, the P'_{gly} of hAQP1-injected oocytes [$(8.91 \pm 0.74) \times 10^{-6}$ cm/s, $n = 43$] decreased to $(1.25 \pm 0.31) \times 10^{-6}$ cm/s, $n = 10$, and P'_{gly} of hAQP2-injected oocytes [$(9.11 \pm 0.42) \times 10^{-6}$ cm/s, $n = 26$] decreased to $(1.52 \pm 0.28) \times 10^{-6}$ cm/s, $n = 10$. Under these conditions, it was impossible to conclude to a only specific chemical interaction of DEPC with histidyl residues of hAQP1 and hAQP2, since both P_f and P'_{gly} were totally inhibited. This is why we performed the site-directed mutagenesis on the histidyl residues of hAQP1 supposed to be the most accessible to Cu²⁺, the inhibitory action of which seemed more specific (see below).

Effects of *p*CMBS on P'_{gly}

All the hAQP1 mutants (C189S, H180A and H209A) raised oocyte P'_{gly} to values similar to those of the wild hAQP1 (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 hAQP1, hAQP1-C189S, -H180A and -H209A, respectively.

The inhibitory effect of 2 mM *p*CMBS on P'_{gly} , already observed in our previous work in hAQP1 cRNA-injected oocytes [2], was conserved for the two

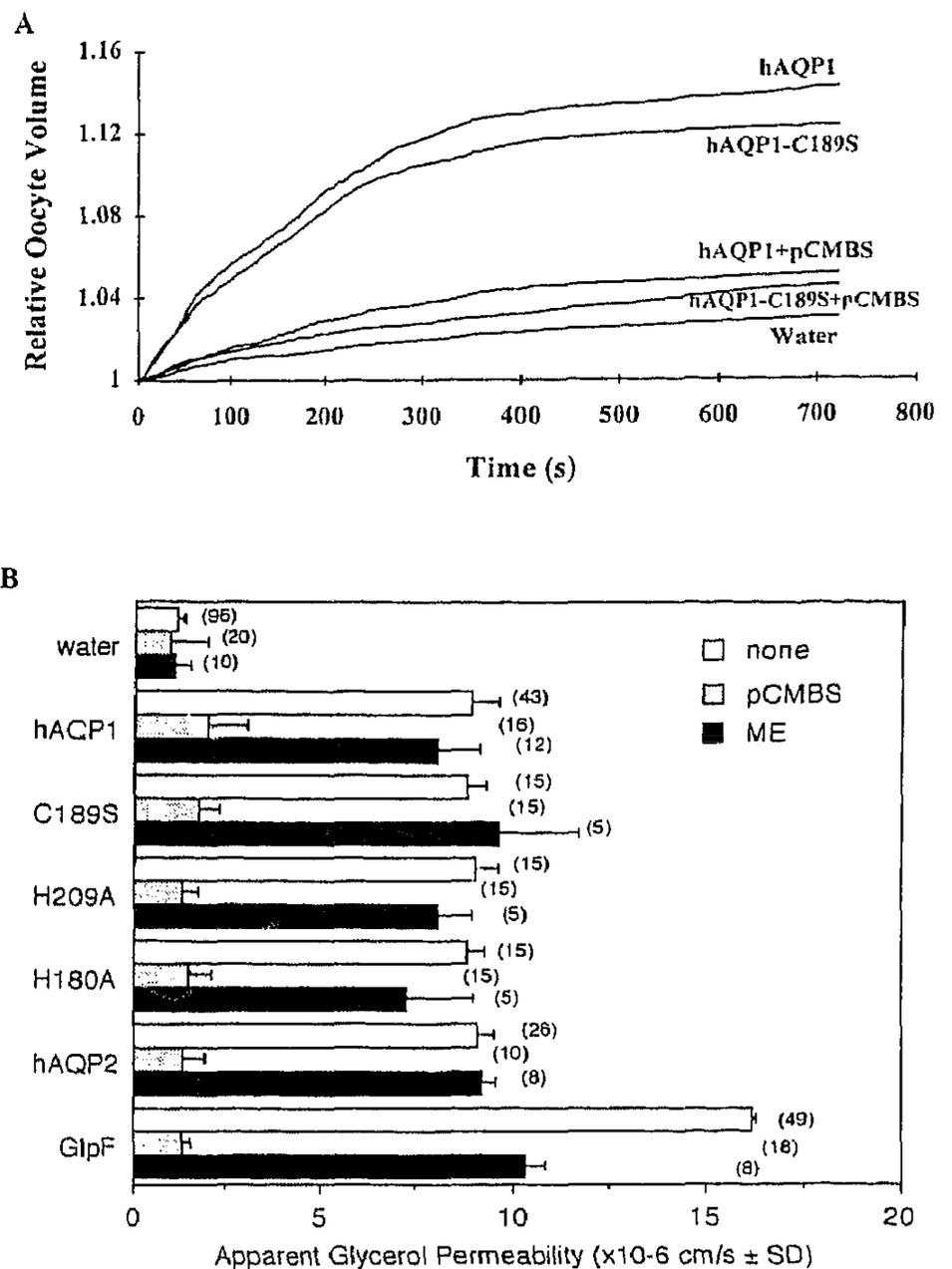


Fig. 2A,B Effect of *p*-chloromercuribenzenesulphonate (*p*CMBS) on the apparent glycerol permeability (P'_{gly}). **A** Time course of osmotic swelling of individual oocytes injected with either water or 10 ng of cRNA encoding human aquaporin 1 (hAQP1) or the mutant hAQP1-C189S. After a 1-h preincubation in the absence or presence of 2 mM *p*CMBS in standard Barth's buffer, oocytes were transferred into fivefold diluted Barth's buffer adjusted to 200 mosmol/l with glycerol. **B** P'_{gly} values of oocytes injected with water and wild or mutant hAQP1, hAQP2 and bacterial glycerol permease facilitator (GlpF). The effect of *p*CMBS was tested as described in A and the reversibility of inhibition by *p*CMBS was measured after a 15-min incubation in the presence of 5 mM β -mercaptoethanol (ME). P'_{gly} values were calculated from initial rates of oocyte volume increase as described in Materials and methods. Bars are means \pm SD (number of determinations in parentheses)

hAQP1 His mutants (Fig. 2) and, more interestingly, was also conserved for the hAQP1-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 hAQP2 facilitates glycerol entry in hAQP2-injected oocytes (Fig. 2), as does hAQP1, and this was also confirmed

by the [^3H]glycerol uptake: P'_{gly} of control = $0.35 \pm 0.048 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{oocyte}^{-1}$ ($n = 48$) and P'_{gly} of hAQP2 = $1.98 \pm 0.18 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{oocyte}^{-1}$ ($n = 18$). The inhibition by *p*CMBS of P'_{gly} 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'_{gly} of GlpF-injected oocytes was strongly inhibited by 2 mM *p*CMBS (Fig. 2), as already described by Maurel et al. [17] in [^{14}C]glycerol uptake experiments. GlpF does not possess the Cys₁₈₉ 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 HgCl₂, even in the absence of the Cys₁₈₉ 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 γTIP does not transport glycerol [P'_{gly} of γTIP was $(1.61 \pm 0.19) \times 10^{-6} \text{ cm/s}$ ($n = 5$) versus $(1.4 \pm 0.34) \times 10^{-6} \text{ cm/s}$ ($n = 5$) in control oocytes].

Effects of CuSO₄ on P'_{gly}

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 Cu²⁺.

As shown in Fig. 3, the augmented glycerol uptake through hAQP1-H180A and -H209A mutants remained inhibited by 0.05 mM CuSO₄ 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 Cu²⁺ is probably more complex than the interaction of Hg²⁺ with the -SH group of Cys₁₈₉ residue and that the replacement of single His residues is insufficient to prevent the inhibitory action of Cu²⁺. A probable explanation is that Cu²⁺ 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 Cys₁₈₉ residue since the inhibition of P'_{gly} by Cu²⁺ is maintained in the C189S mutant (Fig. 3B).

An inhibitory effect of Cu²⁺ 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'_{gly} of GlpF-injected oocytes was not inhibited by Cu²⁺ (Fig. 3B).

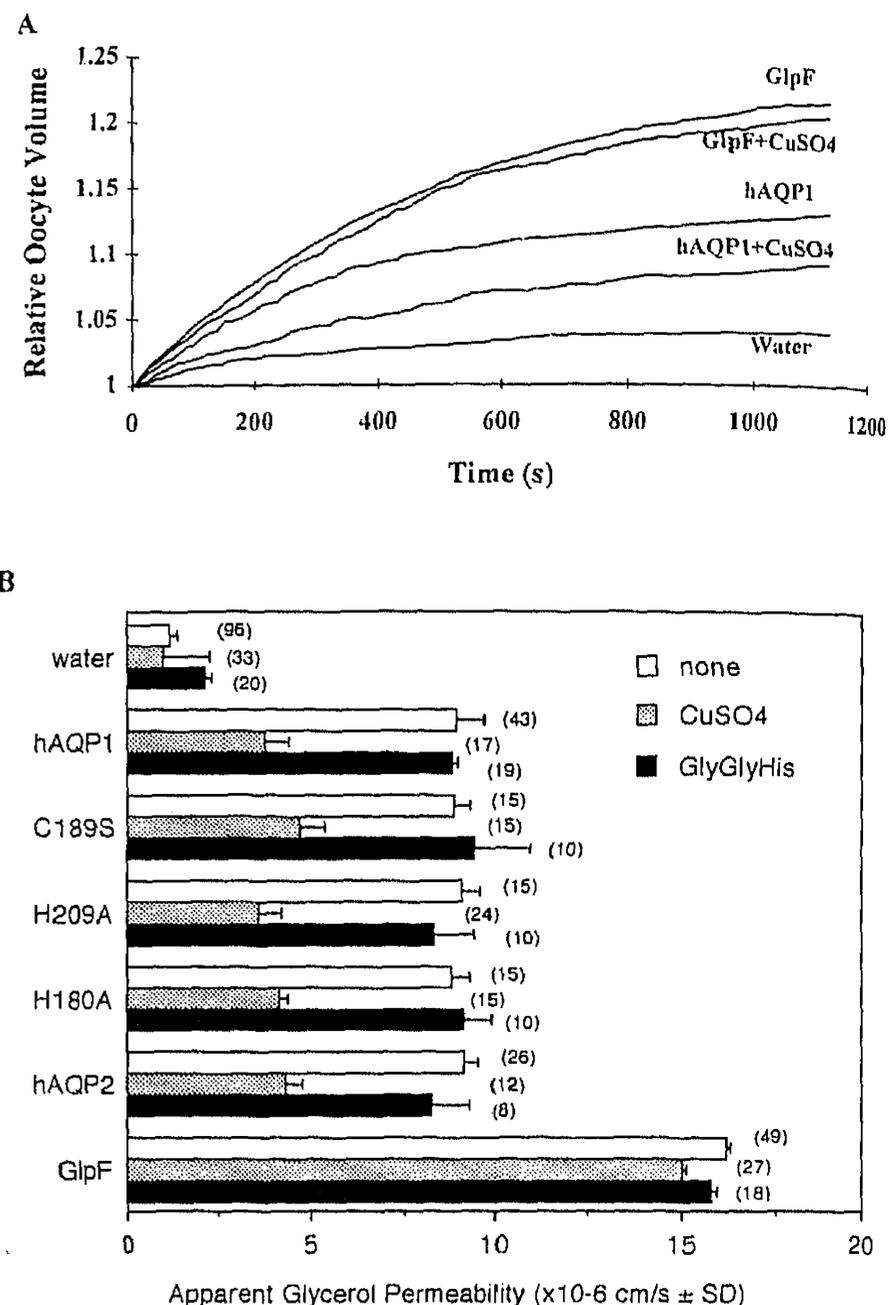


Fig. 3A,B Effect of CuSO₄ on P'_{gly} . **A** Time course of osmotic swelling of individual oocytes injected with either water or 10 ng of cRNA encoding hAQP1 or GlpF. After a 15-min preincubation in the absence or presence of 0.05 mM CuSO₄ in Barth's buffer, oocytes were placed in fivefold diluted Barth's buffer adjusted to 200 mosmol/l with glycerol. **B** P'_{gly} values of oocytes injected with water and wild or mutant hAQP1, hAQP2 and GlpF. The effect of CuSO₄ was tested as described in A and the reversibility of Cu²⁺-induced inhibition was measured after a 15-min incubation in the presence of 1 mM Gly-Gly-His. P'_{gly} values were calculated as described in Materials and methods. Bars are means \pm SD (number of determinations in parentheses)

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 tetrityls, pentitols or hexitols [10], in contrast with AQP1 [2]. Urea 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 Cu²⁺ of glycerol flux through AQP1 and AQP2 is rather specific.

Effects of phloretin on P'_{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 hAQP1, hAQP1-C189S, -H180A, -H209A and hAQP2, respectively. 100% inhibition of glycerol entry by phloretin was observed in GlpF-injected oocytes. It has been reported [15] that 0.25 mM phloretin exerts an inhibitory effect on [3 H]glycerol uptake by rat AQP3 (GLIP).

In conclusion, our study brings some interesting comparative aspects of glycerol and water permeation pathways through hAQP1, hAQP2, the bacterial GlpF and the plant γ TIP by the use of mutants (hAQP1-C189S, -H180A, -H209A) and inhibitors (*p*CMBS, Cu^{2+} and phloretin). hAQP2 was shown to transport glycerol as does hAQP1. GlpF, which is not a water channel, transports glycerol, but the mechanism of glycerol uptake is different from that occurring in hAQP1 and hAQP2. Experiments carried out with another water channel, γ TIP, which does not transport glycerol, demonstrate that certain AQPs are not permeable to glycerol. *p*CMBS-induced inhibition of glycerol transport in hAQP1-C189S-injected oocytes indicates that the Cys residues involved in mercuric inhibition of P'_{gly} and P_r are distinct. Site-directed mutagenesis at two His residues revealed an important role of the hAQP1 His₂₀₉ in water transport, since P_r 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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