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# Zinc Modulation of Water Permeability Reveals that Aquaporin 0 Functions as a Cooperative Tetramer

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We previously showed that the water permeability of AQP0, the water channel of the lens, increases with acid pH and that His40 is required (Németh-Cahalan, K.L., and J.E. Hall. 2000. *J. Biol. Chem.* 275:6777–6782; Németh-Cahalan, K.L., K. Kalman, and J.E. Hall. 2004. *J. Gen. Physiol.* 123:573–580). We have now investigated the effect of zinc (and other transition metals) on the water permeability of AQP0 expressed in *Xenopus* oocytes and determined the amino acid residues that facilitate zinc modulation. Zinc (1 mM) increased AQP0 water permeability by a factor of two and prevented any additional increase induced by acid pH. Zinc had no effect on water permeability of AQP1, AQP4 or MIPfun (AQP0 from killifish), or on mutants of AQP1 and MIPfun with added external histidines. Nickel, but not copper, had the same effect on AQP0 water permeability as zinc. A fit of the concentration dependence of the zinc effect to the Hill equation gives a coefficient greater than three, suggesting that binding of more than one zinc ion is necessary to enhance water permeability. His40 and His122 are necessary for zinc modulation of AQP0 water permeability, implying structural constraints for zinc binding and functional modulation. The change in water permeability was highly sensitive to a coinjected zinc-insensitive mutant and a single insensitive monomer completely abolished zinc modulation. Our results suggest a model in which positive cooperativity among subunits of the AQP0 tetramer is required for zinc modulation, implying that the tetramer is the functional unit. The results also offer the possibility of a pharmacological approach to manipulate the water permeability and transparency of the lens.

## INTRODUCTION

Transition metals, such as zinc and copper, are essential to life. Zinc (Zn), the second most abundant trace element in the body after iron, has been recognized as an essential element for plants and animals for over 60 years (Prasad, 1995, 1996). The major biochemical functions of zinc include its catalytic or structural role in at least 300 zinc metalloenzymes, a structural role in a large number of transcription factors and a role in the maintenance of plasma membrane function. The concentration of zinc in ocular tissues is unusually high compared with that in other tissues (Grahm et al., 2001). Zinc in human ocular tissues is found in retina, choroid, ciliary body, iris, optic nerve, sclera, cornea, and lens, and cataracts are associated with zinc deficiency in fish (Ketola, 1979; Richardson et al., 1985).

AQP0, the major intrinsic protein of the lens (previously known as MIP) is a member of the aquaporin (AQP) family of membrane water channels as are AQP1, AQP4, and MIPfun. AQP0 is found almost exclusively in the lens, whereas AQP1 is found in the brain, kidney, vascular system, and other tissues (King and Agre, 1996) and AQP4 is the most abundant water channel in the brain (Badaut et al., 2002). MIPfun is the AQP0 homologue of the killifish *Fundulus heteroclitus* (Virkki et al., 2001). Expression of AQP0 in oocytes has shown that its water permeability can be modulated by pH and calcium (Németh-Cahalan and Hall, 2000; Németh-Cahalan

et al., 2004) and that external histidines, in particular His40, play a major role in pH sensitivity of AQP0. Zelenina et al. have shown that nickel and copper, but not zinc, reduce the water permeability of AQP3 (Zelenina et al., 2003, 2004), also in a histidine-dependent fashion. Histidine residues are commonly found in consensus copper-binding motifs (Aitken, 1999) and both histidines and cysteines are found in zinc-binding motifs (Vallee and Auld, 1990). Because of this connection of histidine with the modulation of water permeability of both AQP0 and AQP3 and the association of zinc binding sites with histidine, we investigated the effects of zinc, nickel, and copper on the water permeability of AQP0 expressed in oocytes.

## MATERIALS AND METHODS

### Preparation of Oocytes

Female *Xenopus laevis* were anesthetized, and stage V and VI oocytes removed and prepared as previously described (Chandy et al., 1997). The day after isolation, oocytes were injected with 10 ng of the appropriate AQP cRNA and maintained in ND96 (in mM, 96 NaCl, 2 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 5 HEPES, pH 7.5, 2.5 Na-pyruvate) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin at 18°C.

### Expression Constructs and cRNA Preparation

The expression constructs for bovine AQP0 and human AQP1 were gifts from P. Agre and G. Preston (Johns Hopkins University,

Abbreviations used in this paper: AQP, aquaporin; MIP, major intrinsic protein.

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Baltimore, MD). The rat AQP4 gene was purchased from American Type Culture Collection (87184) and placed in a similar expression vector. The expression construct for MIPfun was a gift from W. Boron (Yale University, New Haven, CT). cRNA was transcribed in vitro using T3 RNA polymerase (mMESSAGE mMACHINE kit, Ambion).

### Mutant Construction

All mutants were made using the QuikChange site-directed mutagenesis kit (Stratagene). In brief, the mutants were obtained by performing a one-step polymerase chain reaction with a set of two appropriate primers overlapping in the region of the mutation using *PfuTurbo* DNA polymerase. The mutations were confirmed by sequencing (University of Chicago DNA Sequencing Facility).

### Swelling Assay and Measurement of Water Permeability

2 d post-injection, oocyte swelling assays were performed at controlled temperature of 20–21°C by transfer from 100% ND96 to 30% ND96. Before the transfer to 30% ND96 at the experimental pH or Ca<sup>2+</sup> concentration, oocytes were always equilibrated for 5 min in 100% ND96 at the appropriate experimental pH or Ca<sup>2+</sup> concentration. Water permeability, P<sub>f</sub>, was calculated as previously described (Németh-Cahalan and Hall, 2000; Németh-Cahalan et al., 2004) from optical measurements of the increase in cross-sectional area of the oocyte with time in response to a challenge with diluted ND96. Unless otherwise noted, each data point is the average of experiments using nine oocytes from three different batches. The control solution (ctrl) is ND96 with pH 7.5 and 1.8 mM Ca<sup>2+</sup>. Error bars are shown as ± SEM.

### Transition Metal Treatment

Oocytes were soaked in 100% ND96 containing from 0.1 to 2 mM ZnCl<sub>2</sub>, NiCl<sub>2</sub>, or CuCl<sub>2</sub> for 5 min. Then the swelling assay was performed in 30% ND96 containing the same concentration of the appropriate transition metal.

### Controls

Under standard conditions, uninjected oocytes had an average water permeability of 10.1 ± 0.4 μm/s (*n* = 27), and showed no change in water permeability under any experimental challenge including changes in calcium, pH, or transition metal treatment.

### Oocyte Membrane Isolation and Immunoblot Analysis

Total membranes were prepared as described by Preston et al. (1993). In brief, oocytes were resuspended in the hypotonic lysis buffer and lysed by repeatedly vortexing and pipetting the samples. Series of centrifugations followed to obtain the membrane pellets that were resuspended in 10 mM Tris, 150 mM NaCl, pH 7.5, 1% Triton X-100, and protease inhibitors. Aliquots containing the equivalent of a half oocyte of each sample were loaded onto an SDS gel 4–12% and electro transferred to nitrocellulose, incubated with a 1:1,500 dilution of a polyclonal rabbit antiserum raised against AQP0 C terminus (Kalman et al., 2006) and visualized using the Supersignal West Femto detection system (Pierce Chemical Co.).

### Hill Coefficient Curve Fitting

We used the statistical package in Origin (MicroCal) to fit our data to the Hill equation,

$$P_f = \frac{\Delta P_{f\text{Max}} Z_n^n}{(Z_n^n + K^n)} + P_{f0}, \quad (1)$$

where P<sub>f</sub> is the water permeability, ΔP<sub>fMax</sub> is the increment in water permeability induced by zinc, Z<sub>n</sub> is the zinc concentration, *n* is the Hill coefficient, K is the concentration of zinc at which half

of the maximum effect occurs, and P<sub>f0</sub> is the permeability in the absence of zinc. We used both no weighting (all data points weighted equally) and instrumental weighting (data points weighted according to their standard errors, with larger error points counting less). Fig. 2 B shows the fitted curves with statistical parameters for both given in the legend.

### Calculation of Theoretical Factor of Increase

We define the factor of increase as the water permeability (P<sub>f</sub>) under experimental conditions (with zinc) divided by P<sub>f</sub> under control conditions. We subtracted from both P<sub>f</sub>s the basal water permeability of uninjected oocytes (10.1 μm/s). We used the binomial distribution to calculate the fraction of tetramers containing zero, 1, 2, 3, or 4 mutant monomers under the assumption that the probability of incorporation of mutant and wild type is the same, an assumption validated by experimental data. We could then calculate the factor of increase under the assumption that 1, 2, 3, or 4 insensitive monomers are sufficient to render the entire tetramer insensitive to zinc. For a given ratio of mutant cRNA to total cRNA the theoretical factor of increase, *F<sub>t</sub>(i, p)*, is given by the following formula:

$$F_t(i, p) = \frac{\sum_{j=0}^4 B(j, p) f_i(j, i)}{\sum_{j=0}^4 B(j, p)}, \quad (2)$$

where *B(j, p)* is the binomial probability of a tetramer with *j* insensitive monomers when the ratio of insensitive mutant cRNA to total injected cRNA is *p*,

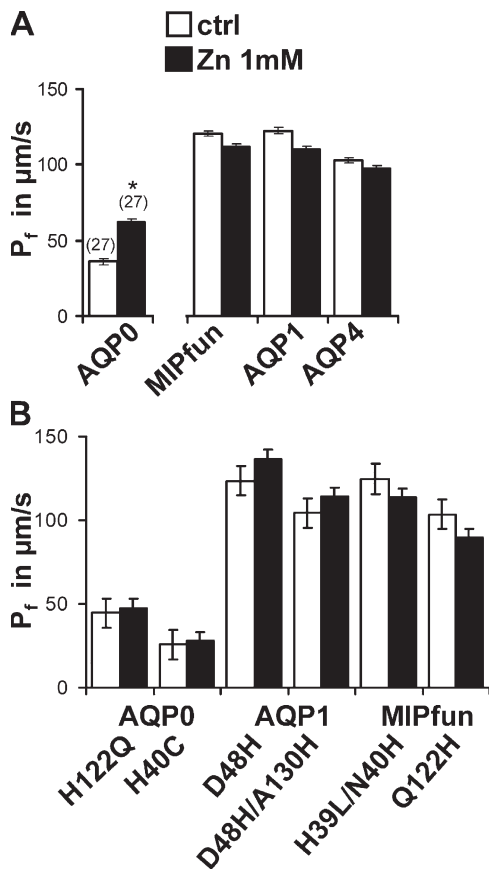
$$B(j, p) = \frac{4!}{j!(4-j)!} p^j (1-p)^{4-j}, \quad (3)$$

and *f<sub>i</sub>(j, i)* is 1 if *i* is greater than *j* and is equal to the maximum factor of increase under control conditions when *i* is less than *j*, where *i* is the number of insensitive monomers in the tetramer and *j* is the number of insensitive monomers required to render the entire tetramer insensitive to zinc.

## RESULTS

### Effect of Zinc on Different Aquaporins (AQP0, MIPfun, AQP1, AQP4)

Zinc (1 mM) increased the P<sub>f</sub> of AQP0 but had no effect on MIPfun, AQP1, or AQP4 (Fig. 1 A). Subtracting the basal water permeability of uninjected oocytes (10.1 μm/s), the factor of increase of AQP0 water permeability induced by zinc is 2.0. There are two accessible histidines (His40 and His122) in the external loops of AQP0, and we have already shown that they are involved in acid pH sensitivity (Németh-Cahalan and Hall, 2000; Németh-Cahalan et al., 2004). AQP1 has no accessible histidines in the external loops, AQP4 presents His130 equivalent to His122 in AQP0, and MIPfun has His39. To evaluate the role of histidines in zinc modulation of AQP0 water permeability we used two point mutants, H40C and H122Q, originally designed to study the role of histidines on the pH effect (Németh-Cahalan et al., 2004). Both mutants, which retained pH sensitivity, completely abolished the zinc-induced permeability increase (Fig. 1 B).

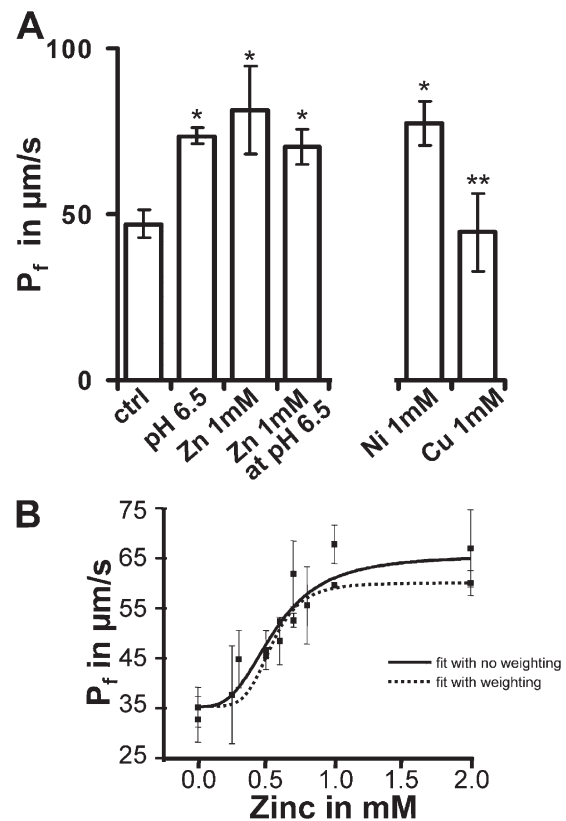


**Figure 1.** (A) Effect of 1 mM ZnCl<sub>2</sub> on the water permeability of AQP0, MIPfun, AQP1, and AQP4. Only AQP0 showed zinc-induced increase in water permeability (from 35.9 ± 2.5 to 62.3 ± 5.5 µm/s). (B) Effect of 1 mM ZnCl<sub>2</sub> on the water permeability of mutants of AQP0, AQP1, and MIPfun. They all lacked the zinc sensitivity. \*, different from control at a significance of  $P < 10^{-5}$  using the Student's *t* distribution. Unless otherwise noted, each data point is the average of experiments using nine oocytes from three different batches.

We previously tested the effect on pH sensitivity of adding histidines to AQP1 and MIPfun (Németh-Cahalan et al., 2004). These mutants (AQP1/D48H, AQP1/D48H/A130H, MIPfun/H39L/N40H, and MIPfun/Q122H), which were pH sensitive, were all insensitive to 1 mM zinc (Fig. 1 B) and also to zinc at a 10-fold higher concentration (not depicted). These results show that histidine-mediated zinc sensitivity does not follow pH sensitivity and indicate a functional requirement for two histidines (His40 and His122) in mediating the zinc-induced modulation of AQP0 water permeability.

#### Acid pH and Zinc

Because histidines almost certainly provide the active binding sites that mediate the effects of both zinc and protons, we expected that modulation of water permeability of AQP0 would reveal a strong interaction between zinc and protons, and this was indeed the case. As shown previously (Németh-Cahalan and Hall, 2000), acid pH by itself

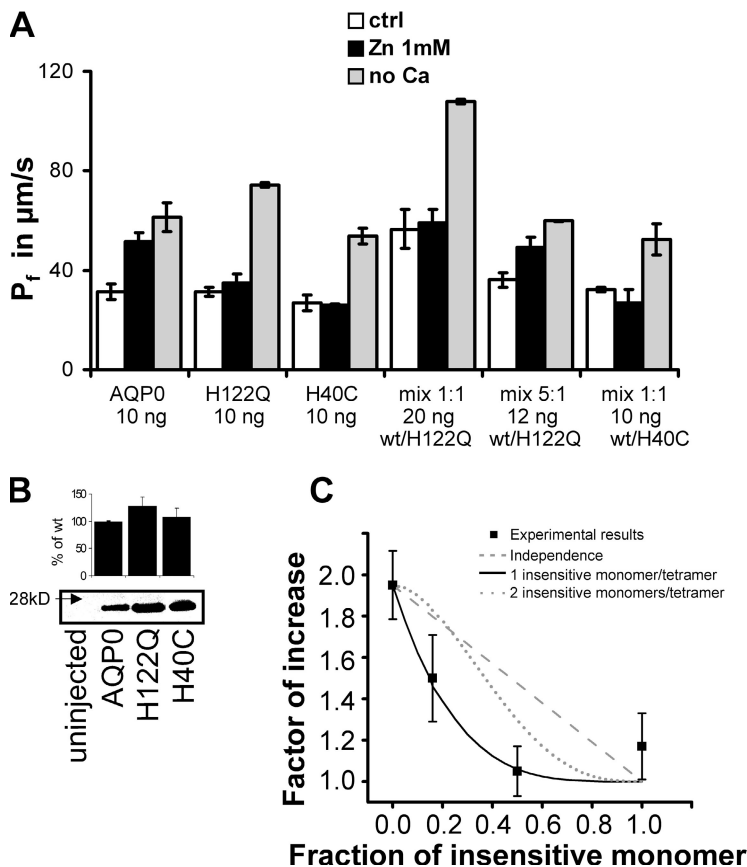


**Figure 2.** (A) Effect of pH 6.5, 1 mM ZnCl<sub>2</sub>, NiCl<sub>2</sub>, or CuCl<sub>2</sub> on the water permeability of AQP0. Zinc did not have any additional effect on water permeability at pH 6.5, suggesting that low pH and zinc act at the same site or sites to increase AQP0 water permeability. Nickel increased  $P_f$  (from 47 ± 4.2 to 77.3 ± 6.6 µm/s). By contrast, copper had no effect. \*, different from control at a significance of  $P < 10^{-5}$ ; \*\*, not significantly different from control  $P > 0.5$  using the Student's *t* distribution. (B) Dose-response curve of the water permeability of AQP0. Fitting with no weighting (continuous line) gives a Hill coefficient of three, suggesting a highly cooperative binding for zinc. Statistical parameters for the no-weighting fit are as follows: reduced  $\xi^2 = 17.1$ ,  $R^2 = 0.9$ ,  $\Delta P_{\text{fmax}} = 30.3 \pm 4.8$ ,  $n = 3.0 \pm 1.2$ ,  $K = 0.6 \pm 0.1$ ,  $P_{f0} = 35.3 \pm 2.7$ . Using instrumental weighting, where the points are weighted according to their standard errors (dotted curve) gives a Hill coefficient of 5. Statistical parameters for the weighted fit are as follows: reduced  $\xi^2 = 1.4$ ,  $R^2 = 0.9$ ,  $\Delta P_{\text{fmax}} = 25.4 \pm 3.0$ ,  $n = 5.1 \pm 0.9$ ,  $K = 0.54 \pm 0.02$ ,  $P_{f0} = 35.3 \pm 2.7$ . While both curves appear to fit the data well, the instrumental fit has considerably better statistics and also gives a greater Hill coefficient. Each data point is the average of experiments using nine oocytes from three different batches.

doubled the water permeability (Fig. 2 A). The factor of increase of AQP0 water permeability induced by acid pH is 1.72. At pH 7.5, 1 mM zinc also elevated AQP0  $P_f$  twofold; but at pH 6.5 with 1 mM zinc, we only saw the doubling of  $P_f$  seen with acid pH or zinc alone. The likely explanation for this lack of additivity is that zinc and protons act at the same site or share a common intermediate state.

#### Other Transition Metals

Several transition metal cations alter the water permeability of another aquaporin, AQP3 (Zelenina et al., 2003, 2004).



**Figure 3.** (A) Effect of 1 mM  $\text{ZnCl}_2$  on the water permeability of coinjected AQP0 and a mutant insensitive to zinc H122Q or H40C. Mix 1:1 represents 10 ng of wild type and 10 ng of mutant H122Q (fraction of insensitive monomer = 10 divided by  $[10+10] = 0.5$ ) and mix 5:1 represents 10 ng of wild type and 2 ng of mutant H122Q (fraction of insensitive monomer = 2 divided by  $[10+2] = 0.166$ ). In both mixtures, the effect of zinc was dramatically diminished but the calcium sensitivity remained intact. Mix 1:1 represents 5 ng of wild type and 5 ng of mutant H40C. (B) Western Blot of uninjected oocyte membranes, AQP0, mutant H122Q, and mutant H40C. A band, lower than the 28 kD marker, was seen in AQP0, H122Q, and H40C and not seen in uninjected oocytes. The bar graphs represent the average of scanned bands from three different experiments. There was no noticeable difference between the wild type and the mutant expression level. (C) Theoretical curves predicting the factor of increase, assuming the monomers behave independently (gray dashed line) or one or two insensitive monomers can block zinc sensitivity (black continuous line and gray dotted line respectively). Experimental results are plotted as filled squares and are well fit by the one insensitive monomer curve. Each data point is the average of experiments using nine oocytes from three different batches.

We tested the effects of two other transition metals, nickel and copper, on AQP0 water permeability. Fig. 2 A shows that nickel increases AQP0 water permeability by a factor of 1.8, but copper has no effect.

#### The Effect of Zinc Is Highly Cooperative

Functional AQP0 is found as a tetramer in the native membrane, so perhaps each monomer must bind zinc for the effect to occur. Fig. 2 B shows a plot of  $P_f$  versus zinc concentration. A fit of these data to the Hill equation (Eq. 1) gave a coefficient of three with no weighting and a coefficient of five with weighting (see statistics in the legend of Fig. 2 B), indicating a very high degree of cooperativity and suggesting that more than one zinc is necessary to obtain the effect and that the binding of one zinc facilitates further zinc binding.

Because the high concentrations of zinc required to obtain an effect suggest a low-affinity site, precluding direct binding studies, we investigated the properties of hetero-tetramers by coinjecting cRNA of a zinc-insensitive mutant with wild-type AQP0. Fig. 3 A shows the  $P_f$  of different mixtures of AQP0 and the insensitive mutants H122Q or H40C. "Mix 1:1" represents 10 ng of each and "mix 5:1" represents 10 ng of wild type and 2 ng of the insensitive mutant H122Q. In the case of mix 1:1, we observed no zinc effect, and mix 5:1 showed a reduced zinc response. Note that the calcium sensitivity (unaffected

by the mutation) remained intact: the  $P_f$  of both wild type and mutant were doubled by no calcium, and the  $P_f$  of mix 1:1 showed the predicted sum of the increases induced by calcium. Parallel coinjection experiments were performed using the second zinc-insensitive mutant, H40C, with exactly the same results. Mix1:1 represents 5 ng of each, and we observed the expected  $P_f$  for 10 ng of total RNA but no zinc effect. Note that the calcium increase remained intact.

To interpret these results, AQP0 and the mutants must express in the membrane at a comparable level. Fig. 3 B shows a Western blot of membranes of uninjected oocytes and oocytes injected with AQP0 or the insensitive mutants H122Q and H40C repeated in triplicate. The specific signal for AQP0 (absent in uninjected oocyte membranes) was of equivalent intensity for both wild type and mutants, suggesting that when we injected the same amount of cRNA of wild type or mutant, the same amount of protein was expressed in the membrane. This conclusion is confirmed by biophysical experiments which show the same level of water permeability when equivalent amounts of wild type and mutant cRNA are injected.

The experimental results of varying the ratio of zinc-sensitive and -insensitive AQP0 are plotted as the factor of increase ( $P_f$  with zinc divided by  $P_f$  without zinc) in Fig. 3 C and can be analyzed to account for the effect of the zinc-insensitive mutant. If there was no interaction

between wild type and mutant aquaporins (independence), the factor of increase would follow the linear combination of the two separate contributions as shown by the gray dashed line in Fig. 3 C. But the experimental values of the factor of increase, calculated from the data in Fig. 3 A and plotted in Fig. 3 C, clearly lie far below this line, indicating that there must be an interaction between mutant and wild type. Provided we assume that wild-type AQP0 and mutant AQP0 express equally and aggregate randomly in heterotetramers, we can predict the factor of increase expected for the various mixtures using Eq. 2. We assess the applicability of these assumptions later in the discussion, but with the provision that they are valid, Eq. 2 fits the data of Fig. 3 C very well with a single insensitive monomer sufficient to eliminate zinc sensitivity of the entire tetramer. In other words, to obtain a  $Zn^{2+}$ -induced increase in the water permeability, every monomer in the tetramer must be zinc sensitive. Note that the curve calculated assuming two insensitive monomers per tetramer are required to suppress zinc sensitivity (gray dotted line) does not fit the experimental data, and the curves requiring three, or four, insensitive monomers for suppression lie above the independence line well to the right (not depicted).

## DISCUSSION

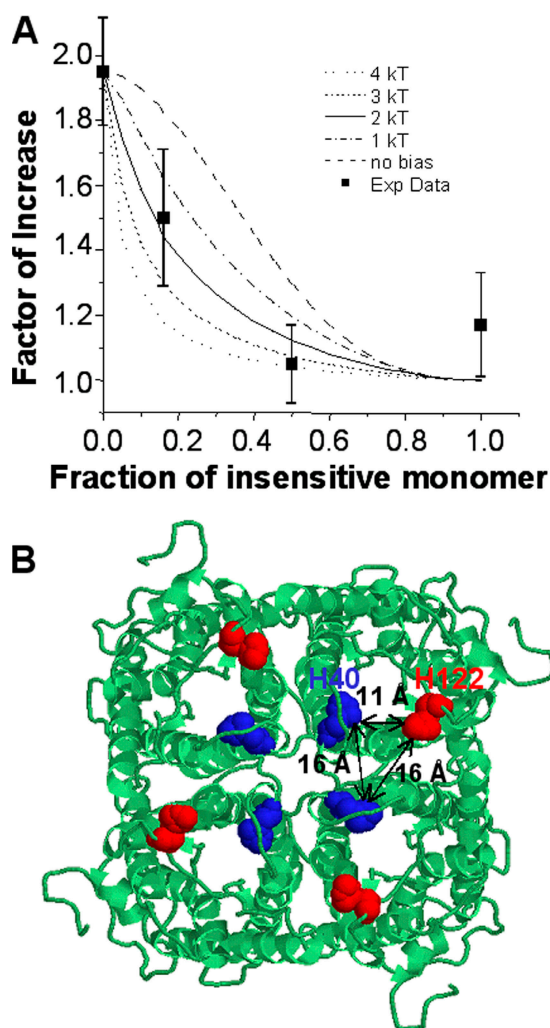
The essential conclusion of this paper is that a cooperative interaction between monomers can account for the modulation of water permeability by  $Zn^{2+}$ . Thus even though each monomer provides its own functional water pore (Preston et al., 1993; Verkman et al., 1995; Sui et al., 2001), the properties of this pore are determined by a cooperative interaction of the entire tetramer. AQP0 is found as a tetramer in both native lens membranes (Zampighi et al., 1982, 1989) and in *Xenopus* oocyte expression system (Chandy et al., 1997) in which all of our experiments were performed. Our results suggest that optimum water permeability requires stabilization of the water pore structure by the cooperative tetrameric structure. Because the monomers fit so closely in the tetramer, the interfaces between them are quite distinctive for each type of aquaporin.

### The Effect of Zinc Is Highly Cooperative

The large Hill coefficient of the zinc titration curve (Fig. 2 B) indicates that zinc may act on the water permeability of AQP0 very much as oxygen acts on hemoglobin to promote cooperative binding. The analogy with hemoglobin suggests that the zinc effect may be mediated by a zinc-induced allosteric cooperative change in structure. Fitting the experimental data in the most disadvantageous fashion for our contention of strong cooperativity (no weighting) gives a Hill coefficient of 3, but the best statistical fit gives a higher Hill coefficient. The data support a strong cooperative effect of zinc modulation of water permeability.

This interpretation is strengthened by the coinjection experiments shown in Fig. 3. These data provide unambiguous proof that the mutant and the wild type interact when expressed in the same oocyte. The simplest type of interaction between wild type and mutant AQP0 is formation of heterotetramers. The analysis presented earlier depends on the assumptions that wild-type AQP0 and mutant AQP0 express equally and aggregate randomly in heterotetramers. We provide two kinds of experimental data that wild type and mutant express equally. First when equal amounts of cRNA of wild type and mutant are injected into separate oocytes, the observed water permeabilities are the same. Second, Western blots of wild-type and mutant protein show that the same amount of protein is expressed in the same number of oocytes under the same conditions. Finally, Chandy et al. (1997) showed that the expression of AQP0 is proportional to the amount of cRNA injected up to  $\sim 30$  ng/oocyte and equal quantities of mutant and wild-type cRNAs produced equal water permeability. Thus the assumption of equal expression is experimentally validated.

This leaves the assumption of random incorporation of mutant protein into heterotetramers. It is overwhelmingly likely that mutant AQP0s form tetramers. Their water permeability and expression levels are the same as those of wild type, and no functional aquaporin water channel has yet been found that does not form tetramers (Lagree et al., 1998; Bron et al., 1999; Mathai and Agre, 1999). Moreover the mutations we are using are not located on the contact surfaces between monomers as revealed in crystal structures of AQP0 and are thus not likely to alter monomer–monomer interactions (Gonen et al., 2004; Harries et al., 2004). Finally the data presented in Fig. 3 demonstrate that wild-type AQP0 and mutant AQP0 must interact. Since the water permeability of oocytes injected with a mixture of mutant and wild-type cRNAs is the same as the sum of the water permeabilities of oocytes separately injected with the same amounts of wild-type and mutant cRNAs alone, the only plausible mode of interaction is formation of heterotetramers. Moreover there is ample precedence for the successful application of exactly the approach we apply here in the analysis of potassium channel stoichiometry. MacKinnon (1991) used the assumptions we use to determine the tetrameric stoichiometry of the potassium channel before either its crystal structure or biochemistry showed a tetrameric structure. And MacKinnon, Aldrich, and Lee used the same assumptions to determine the functional stoichiometry of channel inactivation (MacKinnon et al., 1993). The assumptions of equal expression and random incorporation of mutants and wild-type protein into potassium channel heterotetramers led to conclusions now universally accepted. In our case we already know that AQP0 forms tetramers, and the assumption that mutant and wild type incorporate randomly into heterotetramers fits the experimental data extremely well.



**Figure 4.** (A) Plots of biased binomial predictions of the factor of increase assuming that two insensitive monomers are required to render the heterotetramer insensitive to zinc. Curves are shown for incorporation of mutant AQP0 in the heterotetramer biased by 0, 1, 2, 3, or 4 kT per contact face. Only the 2 kT curve fits the experimental data. (B) Overview of AQP0 tetramer structure. His40 is shown in blue and His122 in red. The tetramer is shown from the extracellular side looking down the pores. The coordinates used to generate the figure were from the published structure of AQP0 (PDB: 6B0P). We used proteinexplorer.org (<http://molvis.sdsc.edu/protexpl/frntdoor.htm>).

Could biased incorporation of mutant into a heterotetramer fit the data equally well? Ding et al. (2005) found that a dysfunctional glycine hinge potassium channel mutant did not suppress the current at cRNA proportions predicted by the binomial distribution but was much less effective than expected. They modified the binomial distribution by adding an energetic penalty for incorporating mutant monomers into a heterotetramer. If, following Ding et al., we add a penalty for incorporation of mutant protein into heterotetramers, we cannot fit the data in Fig. 3 no matter what number of insensitive mutants from 1 to 4 is assumed to render the tetramer

insensitive to zinc (unpublished data). If incorporation of insensitive mutant in the tetramer is energetically favored, only the assumption that two or more insensitive monomers render the tetramer insensitive to zinc can fit the experimental data, and then only if the energetic bias has precisely the right value. Fig. 4 A shows five curves with different energetic biases from 0 to 4 kT per wild-type mutant contact. Only the 2 kT bias curve fits the data. It is important to note that there is no rationale for such a bias, but even in the unlikely event that mutant monomers are favored in heterotetramers by precisely the correct energetic bias, the essential conclusion that the monomers in a tetramer act cooperatively would be unchanged.

#### Cooperative Tetramers in Other Aquaporins

AQP1 is sensitive to mercury and C189 is shown to be the mercury-sensitive residue. But AQP1 shows no cooperativity between monomers in presence of mercury, by coinjection or by construction of dimers of wild type and a mercury-insensitive mutant, C189S, each monomer reacting separately to mercury (Agre et al., 1993; Preston et al., 1994; Shi et al., 1994). In AQP2, the phosphorylation of S256 is essential for its localization in the plasma membrane. Furthermore, the number of phosphorylated monomers in the tetramer is critical for its distribution. Experiments of coinjections of S256A (never phosphorylated) and S256D (pseudo-phosphorylated) show that minimally three monomers have to be phosphorylated for the localization in the plasma membrane, suggesting that the monomers have to interact in the tetramer to induce proper membrane localization (Kamsteeg et al., 2000).

#### Zinc Increases $P_f$ of AQP0 but Not AQP1, MIPfun, or AQP4

In the crystal structures for AQP0 (Gonen et al., 2004; Harries et al., 2004) the distance between His40 and His122 in the same monomer is 11 Å, and the distance between comparable histidines on adjacent monomers is 16 Å (Fig. 4 B). These distances are too long for any two histidines to form simultaneous bonds with zinc, but an ordered water chain could connect histidine side chains with zinc (McCall et al., 2000). Thus, zinc binding to a site made up of two or three histidines (perhaps including bridging waters) could induce a cooperative conformational change of the entire tetramer. The distance measured in AQP1 (PDB: 1FQY) between residues Asp48 and Ala130 is 21 Å and Asp48 and Ala130 do not face each other across the pore as His40 and His122 do in AQP0 (Murata et al., 2000; Sui et al., 2001). We also fitted the Mipfun sequence into the AQP0 structure using DeepView/Swiss-PDB-Viewer. In this case the distance between residues 40 and 122 is around 14 Å but their orientation differs from those in AQP0. No crystal structure is available for AQP4.

## The Transition Metals

There is a large literature on transition metal complexes and the determinants of stability coefficients (Irving, H.M., and R.J.P. Williams. 1953. *J. Chem. Soc.* 3192–3210 (Abstr.); McCall et al., 2000; McCall and Fierke, 2004). These center around the Irving-Williams sequence for complex stability (Mn $\approx$ Fe < Co < Ni < Cu > Zn). It is beyond the scope of this paper to investigate in detail the nature of the zinc binding site, but it is appropriate to point out several possibilities. While zinc and nickel are effective at increasing the water permeability, copper is not. If binding and effect go hand in hand, our results suggest that zinc and nickel bind to the functional site while copper does not. Alternatively binding and effect may not be correlated, and tight binding, say to a single histidine, which copper would achieve if the binding site follows the Irving-Williams sequence, might not promote the same conformational change that distributed binding amongst multiple histidines could. Sorting out these possibilities will be a subject for future investigation.

Finally, the concentration range and pH at which zinc modulates the water permeability of AQP0 suggest that such regulation could well be of physiological importance. The midpoint of the zinc titration curve in Fig. 2 B is 0.5 mM, close to the 0.3 mM average concentration of zinc in the lens, and the local zinc concentration could well exceed the critical value for water permeability increase of 0.5 mM under certain circumstances (Karcioglu, 1982). The lens might thus adjust the local concentration of zinc to modulate water permeability as appropriate. The high degree of cooperativity makes zinc an effective switching agent, and if modulation of water permeability proves to be of physiological importance, the site of zinc action could become an important target for anti-cataract drugs.

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