Copper Inhibits the Water and Glycerol Permeability of Aquaporin-3*

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Aquaporin-3 (AQP3) is an aquaglyceroporin expressed in erythrocytes and several other tissues. Erythrocytes are, together with kidney and liver, the main targets for copper toxicity. Here we report that both water and glycerol permeability of human AQP3 is inhibited by copper. Inhibition is fast, dose-dependent, and reversible. If copper is dissolved in carbonic acid-bicarbonate buffer, the natural buffer system in our body, doses in the range of those observed in Wilson disease and in copper poisoning caused significant inhibition. AQP7, another aquaglyceroporin, was insensitive to copper. Three extracellular amino acid residues, Trp[128], Ser[152], and His[241], were identified as responsible for the effect of copper on AQP3. We have previously shown that Ser[152] is involved in regulation of AQP3 by pH. The fact that Ser[152] mediates regulation of AQP3 by copper may explain the phenomenon of exquisite sensitivity of human erythrocytes to copper at acidic pH. When AQP3 was co-expressed with another AQP, only glycerol but not water permeability was inhibited by copper. Our results provide a better understanding of processes that occur in severe copper metabolism defects such as Wilson disease and in copper poisoning.

Copper is an essential trace element in the human body. It is present in the blood and is incorporated in several vital proteins, such as Cu,Zn-SOD, dopamine β-hydroxylase, ceruloplasmin, cytochrome *c* oxidase, etc. (recently reviewed in Ref. 1). Blood levels of copper are increased in several pathological conditions, such as Wilson disease, Indian childhood cirrhosis, juvenile rheumatoid arthritis, thalassemia, and sickle cell anemia (1–5).

Aquaporin-3 (AQP3)[1] is an aquaglyceroporin expressed in several mammalian tissues including erythrocytes (6–12). It is well established that erythrocytes are permeable for glycerol, but the molecular mechanism for this effect is still obscure. It is also well established that glycerol permeability of erythrocytes is extremely sensitive to copper. We have recently shown that AQP3 water permeability is sensitive to nickel (13). Here we report that copper is a potent inhibitor of AQP3 permeability for glycerol and water. We expressed AQP3 in a human cell line and tested the effect of copper on glycerol and water permeability of the transfected cells. Three extracellular amino acid residues were found to be responsible for the copper sensitivity. We modeled the situation when AQP3 is co-expressed with another AQP, not permeable for glycerol, and found that only glycerol but not water permeability will be inhibited by copper in such cells. The results of this study provide a better understanding of processes that occur in severe copper metabolism defects and copper poisoning.

MATERIALS AND METHODS

cDNA Constructs—The constructs for expression of human AQP3 (wild type and mutants) and the long form of AQP4 have been described previously (13). cDNA fragments encoding full-length AQP3 and AQP4 were obtained by amplification from the human lung QUICK-Clone cDNA library (Clontech). cDNA encoding mouse AQP7 was a kind gift from J. M. Carbrey and P. Agre (Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD). The cDNA fragments were subcloned in-frame into the pEGFP-N2 vector for AQP3 tagged with green fluorescent protein (GFP) at the COOH terminus into pIRES2-EGFP vector (Clontech) for AQP3 and GFP expressed in the same cell as separate proteins and into the pEGFP-C2 vector for AQP4 and AQP7 tagged with GFP at NH₂ termini. The point mutations in extracellular loops of human AQP3 were generated by PCR-based mutagenesis using wild-type cDNA as a template and confirmed by sequence analysis of the whole insert. The transmembrane structure of human AQP3 was predicted using TMHMM Version 2.0 (14).

Cell Culture—The human bronchial epithelial cell line BEAS-2b (subpassages 10–36, European Collection of Cell Cultures, Center for Applied Microbiology and Research, Salisbury, Wiltshire, UK) was cultured on coverslips (Bioptechs, Butler, PA) coated with collagen type I and fibronectin (Sigma) in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (1:1; Invitrogen) containing 0.5 units/ml penicillin, 50 μg/ml streptomycin and supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. On the second day of culture the cells were transiently transfected with cDNA constructs (see above) using CLONfectin (Clontech) according to the manufacturer's protocol. Experiments were performed on the fourth day of culture.

P_f Measurements—The water permeability (P_f) was measured using a method that we recently described in detail (13, 15). The method allows to determine the P_f in individual cells within the cell monolayer and to compare the P_f of cells that do and do not express GFP-tagged proteins. Briefly, the coverslips with the cells were mounted in a closed perfusion chamber (Focht Live Cell Chamber System, Bioptechs) on the stage of a Zeiss 410 invert laser scanning microscope, and the transfected cells were identified by the presence of GFP signal in the plasma membrane. Then the cells were loaded with calcein (Molecular Probes Europe, Leiden, The Netherlands) by incubation for 5 min in solution containing 20 μM calcein-AM (acetoxymethyl ester of calcein) while the

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[1] The abbreviations used are: AQP, aquaporin; GFP, green fluorescent protein; P_f, water permeability; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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FIG. 1. **Water permeability of human AQP3 is down-regulated by copper.** The effect of copper is fast, reversible, and specific. *a*, single cell traces from the water permeability measurements. Fluorescence was monitored in cells loaded with calcein. *Arrows* indicate when the osmolarity was changed from 300 mosM to 200 mosM. The slope of the curves after the change is proportional to the rate of the cell swelling. The difference in the rate of swelling of cell expressing AQP3 (+ *AQP3*) and untransfected cell (− *AQP3*), obvious in control conditions, disappeared when the measurements were repeated in the same cells in the presence of 1 mM CuSO$_4$ and reemerged when CuSO$_4$ was washed out for 1 min. *b*, shown are the mean P_f in transfected cells (*black bars*, *n* = 23) and untransfected cells (*white bars*, *n* = 24) from the experiment described in *a*. *c*, the P_f in neither transfected (*n* = 17), nor untransfected (*n* = 30) cells was changed in the presence of 1 mM lead acetate.
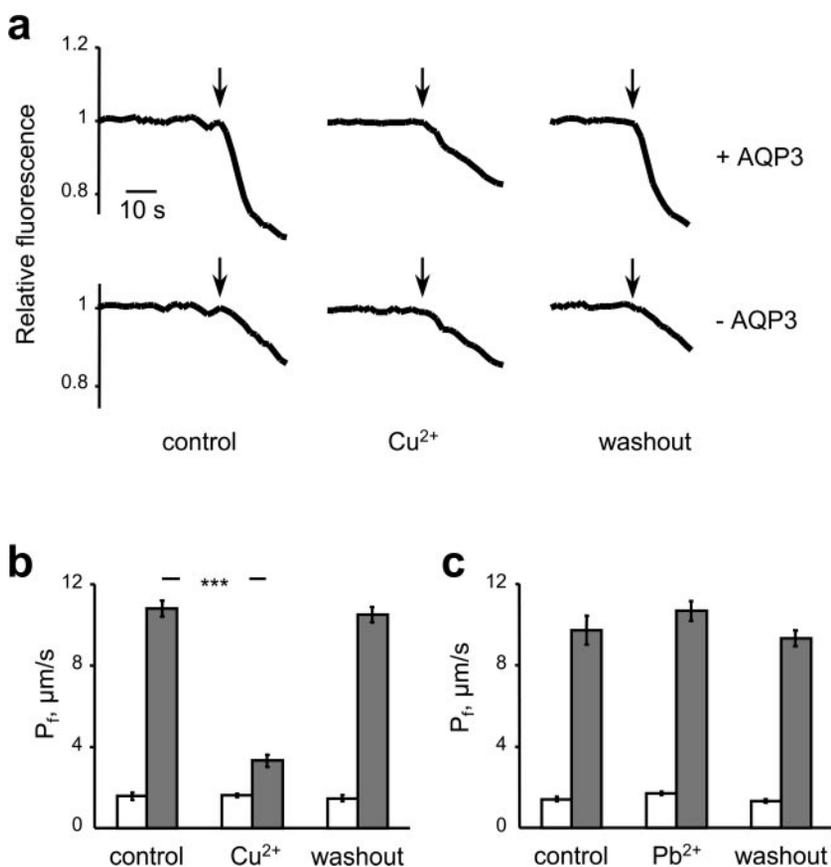
specimen remained on the stage of the microscope. The cells were superfused with isotonic solution (300 mosM) and scanned every 2 s to monitor changes in calcein fluorescence after the solution was switched to hypoosmotic (200 mosM). Isotonic solutions contained in: 137 mM NaCl, 0.9 mM CaCl$_2$, 0.49 mM MgCl$_2$, 4.2 mM KCl, 10 mM buffering compound, pH 7.4. To obtain hypoosmotic solutions, concentration of NaCl was reduced to 87 mM. In experiments with CuSO$_4$ and Pb(CH$_3$COO)$_2$ the pH of the solutions was buffered with Tricine; in experiments with NiCl$_2$ and ZnCl$_2$ the pH of the solutions was buffered with HEPES.

The P_f value was calculated as we described previously (13, 15, 16). The initial slope of the fluorescence curve (about 10 s after the switch of osmolarity) was used in the P_f calculations.

Glycerol Permeability Measurements—To assess the glycerol permeability of the cells (P'_{gly}), we used the same experimental setup as for the water permeability measurements described above. Calcein fluorescence was monitored before and after the switch of superfusion from isotonic solution containing 100 mM mannitol (100 mM mannitol, 87 mM NaCl, 0.9 mM CaCl$_2$, 0.49 mM MgCl$_2$, 4.2 mM KCl, buffering compound 10, pH 7.4) to the same solution where mannitol was replaced by 100 mM glycerol. The initial slope of the fluorescence curve (about 10 s after the switch of osmolarity) was used as a measure of the glycerol permeability.

Data Presentation and Analysis—Data are presented as means ± S.E. Statistical analysis was performed using Student's *t* test. A difference of *p* < 0.05 was considered statistically significant.

RESULTS

Water Permeability of AQP3 Is Down-regulated by Copper—Experiments were performed in a human epithelial cell line BEAS-2b that was transiently transfected with human AQP3 tagged with GFP at the COOH terminus to identify the cells that expressed AQP3. In control conditions, the water permeability in these cells was significantly higher compared with the surrounding untransfected cells (Fig. 1, *a* and *b*). When the measurements were repeated in the presence of 1 mM CuSO$_4$, the water permeability of AQP3-positive cells decreased almost to the level of untransfected cells. After copper washout, the water permeability of AQP3-positive cells increased to the level

observed in control conditions. In the same type of experiment, the water permeability of AQP3-positive cells was insensitive to lead acetate.

Glycerol Permeability of AQP3 Is Down-regulated by Copper—Glycerol permeability of BEAS-2b cells that expressed AQP3 was significantly higher than permeability of untransfected cells (Fig. 2). The rate of swelling in glycerol-containing solution was significantly decreased in AQP3-positive cells in the presence of 1 mM CuSO$_4$. The same effect was observed in the presence of NiCl$_2$. However, the glycerol permeability of AQP3-positive cells was not influenced by divalent zinc and lead. Neither of the metals influenced the glycerol permeability of untransfected cells.

Both water permeability and glycerol permeability of AQP3-positive cells were down-regulated by copper dose-dependently (Fig. 3*a*). Even after the measurements in the presence of 5 mM CuSO$_4$, the effect of copper was to a large extent reversible.

Water and Glycerol Permeability of Cells Transfected with AQP3 Alone or Co-transfected with Another AQP—In the cells transfected with AQP3 alone, the permeability for water and glycerol was down-regulated to the same extent at each concentration of copper (Fig. 3*a*). However, in cells that co-expressed AQP3 and AQP4 only glycerol permeability was affected by copper (Fig. 3, *b* and *c*). In these experiments, we used an AQP3 construct that expressed AQP3 and GFP in the same cell as separate proteins. As we have shown previously (13), the cells successfully transfected with this construct can be identified by GFP signal distributed homogenously throughout the cytoplasm. In these cells, permeability to both water and glycerol was dramatically decreased by copper (Fig. 3, *b* and *c*, *AQP3*). Another construct used in these experiments encoded AQP4 tagged with GFP. The cells successfully transfected with this construct were identified by a distinct GFP signal in the plasma membrane of the cells. The cells expressing AQP4 only were not permeable for glycerol, and their water permeability
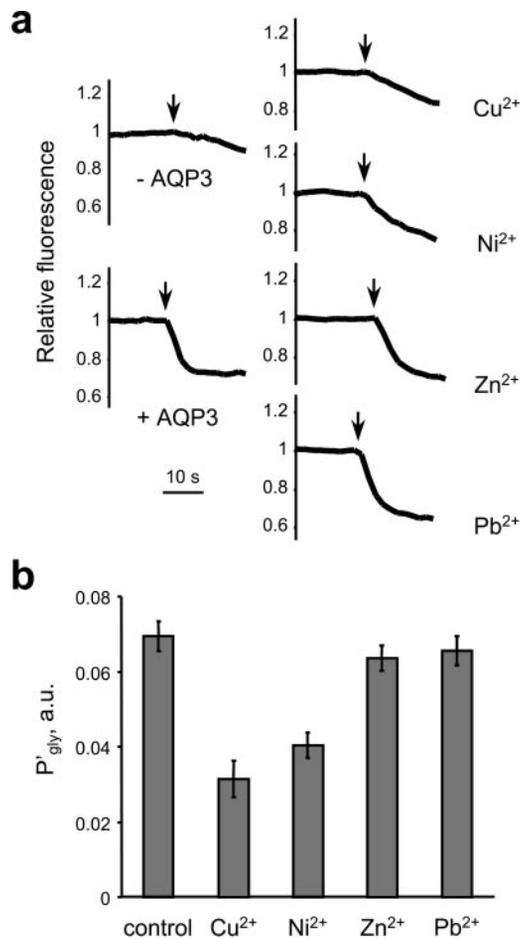
FIG. 2. **Glycerol permeability of human AQP3 is down-regulated by copper and nickel but is not sensitive to zinc and lead.** *a*, single cell traces from the glycerol permeability measurements. Fluorescence was monitored in cells loaded with calcein. *Arrows* indicate when mannitol solution was changed to isoosmotic glycerol solution. The slope of the curves after the change is proportional to the rate of the cell swelling. The *left panel* shows the difference in the rate of swelling of cell expressing AQP3 (+ AQP3) and untransfected cell (− AQP3). On the right panel, shown are the traces from transfected cells in the presence of 1 mM CuSO₄, NiCl₂, ZnCl₂, and lead acetate. *b*, shown are the mean glycerol permeabilities in transfected cells from the experiment described in *a* (*n* = 15–35).

was not influenced by copper (Fig. 3, *b* and *c*, *AQP4*). Cells co-transfected with a mixture of AQP3 and AQP4 constructs were recognized by the presence of GFP signal both in cytoplasm and in the plasma membrane. In these cells, the water permeability was virtually insensitive to copper, but glycerol permeability was dramatically decreased when the cells were exposed to copper (Fig. 3, *b* and *c*, *AQP3+AQP4*).

Effect of the Heavy Metals on the Water Permeability of AQP3 Measured in Carbonic Acid-Bicarbonate Buffer—Because buffering compounds may to a large extent chelate divalent ions, the effective concentration of the metals in buffers such as Tris, Tricine, and HEPES is substantially lower than the level at which it was added. We examined the effect of copper, nickel, lead, and zinc dissolved in carbonic acid-bicarbonate buffer and found that the water permeability of AQP3-positive cells was down-regulated at very low concentrations of copper and nickel but still was not influenced by zinc and lead (Fig. 4). Copper had a significant inhibitory effect at concentrations at least 5-fold lower than nickel.

Unfortunately, running the measurements in bicarbonate buffer is extremely difficult because of abundant gas bubble formation in the chamber. The rest of the experiments were
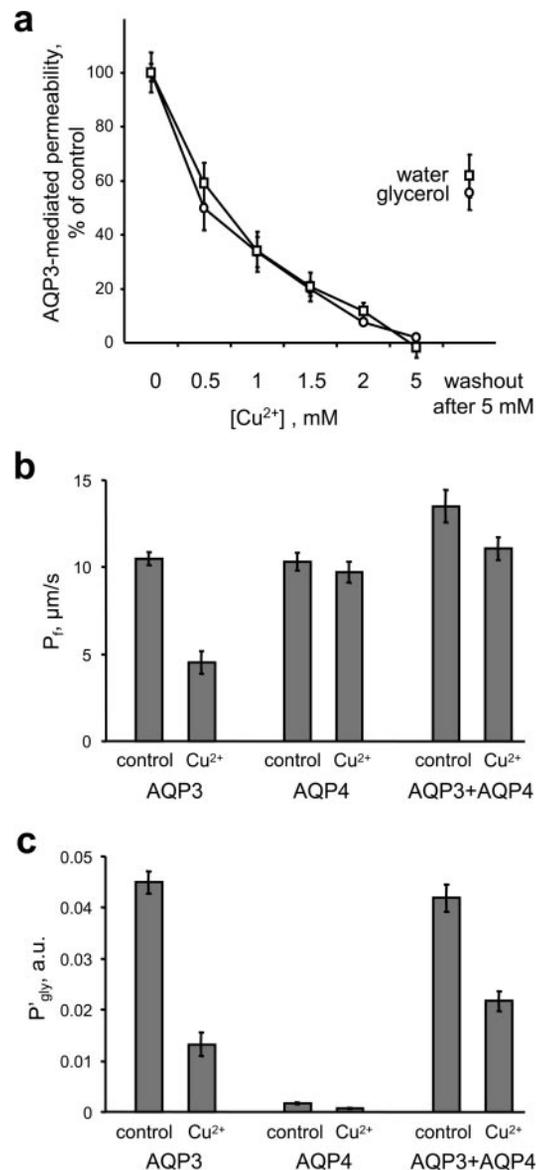

FIG. 3. **In cells where AQP3 is expressed alone, copper down-regulates water and glycerol permeability in a similar fashion; when AQP3 is co-expressed with another AQP, only glycerol permeability of the cells is affected.** *a*, cells transfected with AQP3. The measurements of water and glycerol permeability were performed in parallel experiments. The difference of permeability of transfected and surrounding untransfected cells was normalized to the corresponding control. Each point represents average data from 14–51 cells. *b* and *c*, cells transfected with AQP3, AQP4, or co-transfected with AQP3 and AQP4 (*n* = 4–16 cells). In cells expressing AQP3 and AQP4 together the water permeability (*b*) is only slightly affected by copper; the glycerol permeability (*c*) is decreased dramatically in these cells.

therefore performed in Tricine and HEPES buffer.

Amino Acid Residues Involved in Regulation of AQP3 by Copper—We have previously identified amino acid residues involved in the regulation of AQP3 by nickel (13). We examined these mutants in experiments with copper and found that the same residues are involved in the effect of this metal on the water permeability of AQP3 (Fig. 5).

Water and Glycerol Permeability of AQP7—BEAS-2b cells were transiently transfected with mouse AQP7 tagged with GFP at the NH₂ terminus. Permeability to water and glycerol were measured in the absence and in the presence of copper and nickel. Neither of the ions influenced the permeability of AQP7-positive cells (data not shown).

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FIG. 4. **In bicarbonate buffer, very low concentrations of copper and nickel inhibit human AQP3, whereas lead and zinc have no effect.** Copper is a more potent inhibitor of AQP3 than nickel. Data are presented as difference between P_f in transfected cells and surrounding untransfected cells ($n = 9–67$ cells).
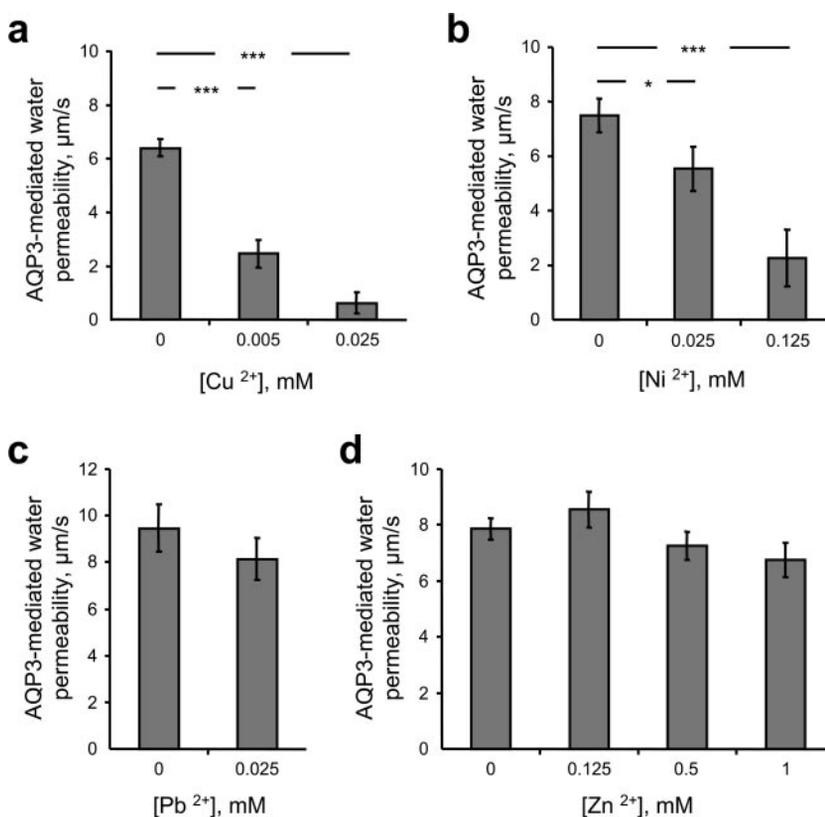
DISCUSSION

Copper is a trace element essential to life. Its role in enzyme catalysis and gene expression is well established. More recently it has been demonstrated that copper can regulate brain ligand-gated ion channels (17, 18). Here we show that copper is also a potent reversible inhibitor of both water and glycerol transport of the aquaglyceroporin AQP3. We show that in carbonic acid-bicarbonate buffer, the natural buffer system in our body, low concentrations of copper are sufficient to dramatically decrease AQP3 permeability. These concentrations of copper are lower than copper levels observed in human blood in Wilson disease (19) and more than an order of magnitude lower than those that can be observed in copper poisoning (20).

We show that the effect of copper on AQP3 is rapid and reversible. Copper does not need to enter the cells to exert its effect on AQP3. Three amino acid residues in the second and the third extracellular loops, Trp[128], Ser[152], and His[241], were shown to be involved in the copper-mediated AQP3 inhibition. The same amino acids were previously shown to mediate nickel effect on AQP3 (13). One of these, Ser[152], is also involved in regulation of AQP3 by extracellular pH. The fact that Ser[152] is the molecular target both for protons and copper may explain the phenomenon of exquisite sensitivity of human erythrocytes to copper at acidic pH (21) and emphasizes the importance of immediate treatment of metabolic acidosis that occurs in copper poisoning (22).

AQP3 inhibition by copper is principally different from inhibition by mercury, which is also a divalent heavy metal. Mercury affects AQPs by acting on cysteine residues of the water channels. The mercury-sensitive cysteine in AQP3, Cys[11], is located in the NH_2-terminal part of the protein (23). Thus, mercury has to penetrate the cell membrane to reach its target, which makes the mercury effect relatively slow. As discussed above, copper acts on AQP3 extracellularly. Another difference between mercury and copper is in the nature of the bonds formed by the metals with AQP3. Mercury binds to proteins

covalently. As a consequence, the effect of mercury is only reversible upon treatment of the cells with a reducing agent, such as β-mercaptoethanol. In contrast, copper forms coordination bonds with proteins. As a result, the effect of copper is to a large extent reversible by simple washout of the metal ions. The speed and reversibility of copper and nickel effects make these metals very convenient as test tools in functional studies in cells where AQP3 is co-expressed with other water transporters. Effects of copper and nickel are highly specific; neither of the AQPs tested in our previous study (13) and the present study proved to be sensitive to these metals, and several other heavy metals were ineffective with regard to the permeability of AQP3.

Copper down-regulated the water and glycerol permeability of AQP3 to the same extent. This is in apparent contrast to the differential regulation of water and glycerol permeability of AQP3 by pH (24). The parallel effects of copper on water and glycerol permeability of AQP3 suggest that copper blocks the pore of AQP3 rather than changes the conditions in the pore. Identification of amino acid residues that are involved in the copper and nickel effect should provide valuable information for future studies of the secondary structure of AQP3.

It is well documented that glycerol permeability of human red blood cells is decreased in the presence of copper (11, 21, 25), but the mechanisms of the copper action have been unknown. In red blood cells AQP3 is co-expressed with AQP1, which is not glycerol permeable. Our co-expression study shows that the consequences of AQP3 down-regulation by copper will depend on whether AQP3 is the only water channel in a particular cell or whether it is co-expressed with other water channels. In red blood cells, kidney collecting duct, bronchial epithelium, and colon AQP3 are co-localized with other AQPs (6, 8, 9, 11, 26). We found that when AQP3 is expressed alone, both water and glycerol permeability of the cells is down-regulated by copper. However if AQP3 is co-expressed with another AQP, only permeability for glycerol is affected.
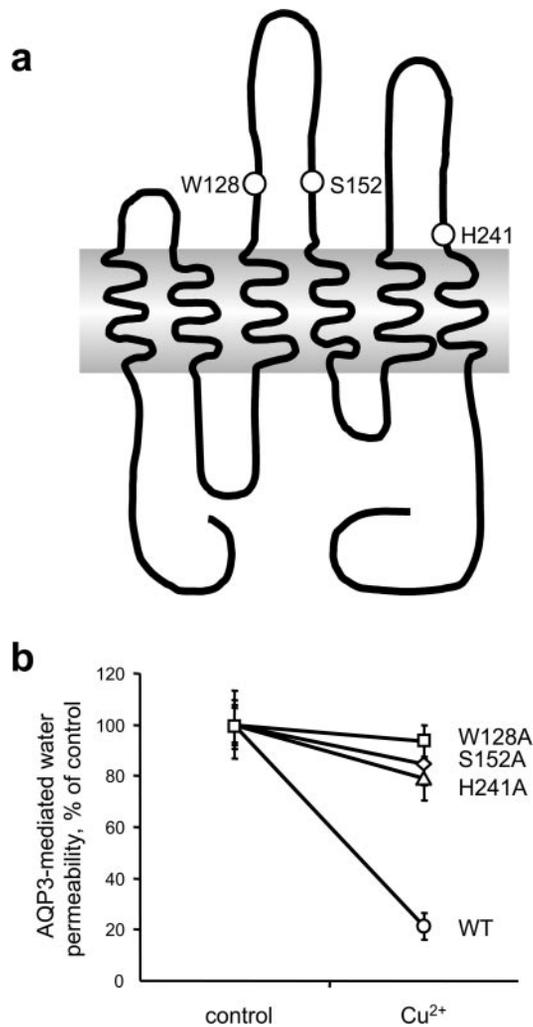
FIG. 5. **Amino acid residues involved in regulation of AQP3 by copper.** *a*, Trp[128], Ser[152], and His[241] are situated in putative extracellular loops of human AQP3. *b*, in contrast to cells expressing wild-type AQP3 (*WT*), the P_f in cells expressing AQP3(W128A), AQP3(S152A), or AQP3(H241A) was not decreased by 1 mM CuSO$_4$. Data are presented as difference between P_f in transfected cells and surrounding untransfected cells normalized to corresponding control (*n* = 15–47 cells).

Copper is still widely used in agriculture, mostly to control bacterial and fungal diseases of fruits and vegetables. It is also used as a herbicide in irrigation and municipal water treatment systems. Accidental and suicidal copper poisoning is not rare, and manifestations of acute copper poisoning are observed in kidney and red blood cells, the tissues where AQP3 is expressed. Copper is also widely used in water distribution systems. Considerable amounts of copper can be present in the tap water, especially in pipes that undergo an internal corrosion (27, 28). Copper present in drinking water can cause nausea, vomiting, abdominal pain, and diarrhea (29). Chronic dietary copper intake may lead to liver damage (30). Children are especially susceptible to the poisoning, probably because of an imbalance between absorption and excretion of copper in the early postnatal period (31). Severe impairment of the hepatic copper excretory function, observed in Wilson disease, may lead to acute hemolytic crisis (32) similar to that in acute copper poisoning.

The copper sensitivity of AQP3 will likely contribute to some of the clinical manifestations of acute and chronic copper intoxication. It is known that glycerol has a protective effect on red blood cells (33). The mechanisms by which glycerol, taken up by red blood cells, can have a protective effect is still incompletely understood, but it has been suggested that the effect of glycerol as a scavenger of hydroxyl radicals may be of importance. We speculate that glycerol may also play a role in the volume control of red blood cells. Intravascular hemolysis is one of the chief manifestations in copper poisoning. We suggest that inhibition of glycerol permeability of erythrocytes caused by inhibition of AQP3 by copper is one of the causes of the impaired erythrocyte structural integrity.

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