Electrostatic attraction of weak monoacid anions increases probability for protonation and passage through aquaporins

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A positive electrostatic field emanating from the center of the aquaporin (AQP) water and solute channel is responsible for the repulsion of cations. At the same time, however, a positive field will attract anions. In this regard, l-lactate/lactic acid permeability has been shown for various isoforms of the otherwise highly water and neutral substrate selective AQP family. The structural requirements rendering certain AQPs permeable for weak monoacids and the mechanism of conduction have remained unclear. Here, we show by profiling pH-dependent substrate permeability, measurements of media alkalinization, and proton decoupling that AQP9 acts as a channel for the protonated, neutral monocarboxylic acid species. Intriguingly, the obtained permeability rates indicate an up to 10 times higher probability of passage via AQP9 than given by the fraction of the protonated acid substrate at a certain pH. We generated AQP9 point mutants showing that this effect is independent from properties of the channel interior but caused by the protein surface electrostatics. Monocarboxylic acid-conducting AQPs thus employ a mechanism similar to the family of formate-nitrite transporters for weak monoacids. On a more general basis, our data illustrate semiquantitatively the contribution of surface electrostatics to the interaction of charged molecule substrates or ligands with target proteins, such as channels, transporters, enzymes, or receptors.

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2 The abbreviations used are: AQP, aquaporin; NPA, Asn-Pro-Ala region; ar/R, aromatic/arginine constriction; GlpF, glyceral facilitator; FNT, formate-nitrite transporter; DNP, 2,4-dinitrophenol.

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brain, in particular at the blood-brain barrier, in glia cells, and in catecholaminergic neurons, suggests physiological functions in energy provision in the form of ketone bodies, and in the protection of neurons in pathological ischemia by channeling l-lactate (27, 28).

Here, we show that the positive surface electrostatics of AQP9 is crucial for attracting monoacid anions, protonation of the substrate, and passage of the neutral species. We further found that protonation efficiency increased with substrate size compensating impediments to mobility of the larger molecules. As a consequence, at neutral pH, all tested monoacid substrates, i.e. acetic acid, l-lactic acid, and particularly β-hydroxybutyric acid, passed AQP9 at rates that suggest physiological relevance.

Results
Permeability of AQP9 for lactate/lactic acid increases with lower pH

Initially, we determined the pH profiles of lactate/lactic acid permeability of the human aquaglyceroporin AQP9 and murine AQP7, for which monoacid permeability has not been described before. We expressed AQP9 and AQP7 in yeast, prepared osmosensitive protoplasts, and subjected them to a 50 mM inwardly directed gradient of lactate/lactic acid in the range of pH 3–7. Cellular volume changes were monitored via changes in the light scattering intensity (Fig. 1A) (29). In a first, rapid phase (<15 s), osmotic water efflux via the expressed AQP and across the plasma membrane decreases the cell volume as indicated by an increase in light scattering. In a second, slower phase (>15 s), volume will be regained because of solute influx and a secondary osmotic inflow of water resulting in a decrease in the light scattering intensity. Lactate/lactic acid permeability of AQP9-expressing yeast cells increased toward lower pH (Fig. 1A, left panel), whereas with AQP7 hardly any permeability was detectable at all pH conditions (Fig. 1B, left panel). To test for pH effects on the AQP protein integrity and to relate the lactate/lactic acid permeability of AQP9 and AQP7 to a common, similarly sized and neutral substrate, we determined glycerol permeability. Glycerol has a molar mass of 92 g mol⁻¹, and lactic acid has a molar mass of 90 g mol⁻¹ at a similar van der Waals volume (88.0 versus 81.6 Å³; Table 1). However, with a pKₐ of 14.2, glycerol will remain neutral in aqueous solution independent of the pH. AQP9 was functional at all tested pH conditions, and glycerol permeability decreased by 45% with increasing buffer acidity (Fig. 1A, right panel). AQP7 was highly permeable for glycerol at a pH of 5 and greater but exhibited a sudden loss of functionality at pH 4 and below (Fig. 1B, right panel). This pH dependence of AQP7 has not been shown before and is probably due to protonation of critical aspartate (pKₐ = 3.9) and/or glutamate residues (pKₐ = 4.3). From the light scattering traces, we extracted the permeability coefficients (Pₛₒ₁) by exponential fittings of the second, solute diffusion phase (6). We plotted the permeability coefficients against the pH and obtained sigmoidal curves for lactate/lactic acid permeability of AQP9, whereas the curve for AQP7 was flat (Fig. 1C, left panel). The lactate/lactic acid permeability rates for AQP9 were maximal at the most acidic assay condition, pH 3. Here, the fraction of neutral lactic acid is 0.9 of the total lactate, and the observed permeability coefficient (0.55 ± 0.10 μm s⁻¹) matched that of glycerol (0.48 ± 0.06 μm s⁻¹; Fig. 1C, right panel). A plot depicting the permeability ratio for lactate/glycerol as a function of pH is shown in supplemental Fig. S1. The pH profile suggests passage of the neutral, protonated lactic acid form via AQP9. If the charged lactate anion was the permeating species, a reversed sigmoidal curve would have resulted with decreasing rates toward lower pH. Quite unexpectedly, comparison of the permeability rates with the fraction of available neutral lactic acid at each tested pH (Fig. 1C, dotted line) revealed a shift by 0.6 log units, i.e. the substrate permeability is 10⁴⁺ (four) times higher than predicted. Because of full blockage of AQP7 at pH 3 and small residual functionality of
14% at pH 4 (Fig. 1C, right panel), the data for AQP7 could be analyzed only at pH 5, 6, and 7. In this range, AQP7 exhibited 2–4-fold higher glycerol permeability than AQP9 (Fig. 1C, right panel). However, permeability for lactic acid was at best one-eighth that of AQP9 (Fig. 1C, left panel, and ratio plot in supplemental Fig. S1). This and the offset between the existing lactic acid fraction in the buffer and the observed permeability rates clearly hint at a specially adapted mechanism of AQP9 to facilitate lactate/lactic acid permeability.

**AQP9 channels neutral lactic acid independent of the transmembrane proton gradient**

The protonation state of the AQP9–passing substrate species directly affects the proton concentration in the buffer. Passage of the neutral, protonated lactic acid species via AQP9 would lead to alkalization of the media caused by a loss of protons, whereas uptake of the lactate anion would lead to acidification (30). Hence, we monitored pH changes in the weakly buffered external solution using a pH electrode during lactate/lactic acid uptake (Fig. 2A). AQP9-expressing yeast cells steadily alkalized the buffer confirming uptake of the protonated lactic acid substrate, whereas non-expressing cells marginally alkalized the buffer.

We next addressed the question whether AQP9 has adopted secondary transporter-like functions by making use of a transmembrane proton gradient. Therefore, we measured lactic acid influx via AQP9 at an external of pH 4, i.e. a 1000:1 inward proton gradient (assuming a cytosolic pH of 7), with and without addition of a protonophore, 2,4-dinitrophenol (DNP) (31). Despite disruption of the proton gradient by DNP, the lactic acid permeability of AQP9 remained unaltered (Fig. 2B). The data indicate that AQP9 acts as a channel, and permeability rates are determined solely by the availability and transmembrane gradient of neutral lactic acid.

**The extracellular protein surface of AQP9 is positively charged**

Our observation that AQP9 conducts lactic acid at a four times higher rate than predicted from the pH-dependent availability of lactic acid, whereas AQP7 exhibited very low lactic acid permeability only at the most extreme acidic pH remained puzzling. We hypothesized that the degree of electrostatic attraction of negatively charged substrate molecules may be different between AQP9 and AQP7 leading to a higher local concentration of lactate/lactic acid at the AQP9 protein surface. Calculation of the Poisson-Boltzmann electrostatics (32) showed a strongly positive extracellular surface of an AQP9 structure model (33), whereas AQP7 was oppositely charged (Fig. 3). To connect the surface properties to specific amino acid positions, we scanned the AQP9 sequence for cationic residues with a particular focus on sites where other AQPs carry non-charged residues (34). This way, we identified three candidate residues (Fig. 3A, top center): His146 located just above the ar/R constriction site and two arginines, Arg51 and Arg53, in the extracellular loop A connecting transmembrane spans 1 and 2 (Fig. 3B). The latter form a central elevation at the extracellular face of the AQP9 tetramer. To evaluate effects of the candidate residues, we calculated the surface charge of the respective AQP9-H146A, AQP9-R51A,R53A, and AQP9-R51E,R53E mutants (Fig. 3A, bottom panels). Although AQP9-H146A showed a small reduction in positive surface electrostatics, AQP9-R51A,R53A appeared fairly neutral, and AQP9-R51E,R53E exhibited a strongly negative protein surface similar in extent to AQP7 (Fig. 3A).

**AQP9 surface electrostatics determines lactic acid permeability**

We generated the AQP9 mutants AQP9-H146A, AQP9-R51A,R53A, and AQP9-R51E,E53E for functional assays in yeast. Western blot analysis confirmed expression of the AQP9 mutants at equal or somewhat greater levels than wild-type AQP9 (Fig. 4A). Next, we assayed for glycerol permeability at neutral pH to evaluate general protein functionality (Fig. 4B). Here, quite unexpectedly, AQP9-R51A,R53A came with a loss of function and had to be eliminated from the test set. AQP9-H146A and AQP9-R51E,E53E conducted glycerol at equal rates as wild-type AQP9 and, hence, were usable for lactic acid permeability assays. AQP9-H146A showed a pH profile of lactic acid permeability (Fig. 4C) identical to that of wild-type AQP9 (Fig. 4C, dashed line). In the AQP9-R51E,E53E mutant, however, lactate permeability turned out to be strongly reduced across the pH 3–7 range (Fig. 4D and supplemental Fig. S1); however, glycerol permeability was
observed and equal to wild-type AQP9 at all pH conditions, indicating protein functionality (Fig. 4D, inset). The very-low lactic-acid conductance in combination with high glycerol permeability mirrors the properties of AQP7 (Fig. 4D) and may be directly attributable to repulsion of lactate anions by the negative surface electrostatics.

Acetic and β-hydroxybutyric acid show shifted permeability profiles

To test for an influence of substrate pK_{a} and size, we determined the pH profiles of AQP9 conductance for acetate/acetic acid (pK_{a} = 4.8; van der Waals volume 55.9 Å³) and β-hydroxybutyrate/β-hydroxybutyric acid (pK_{a} = 4.4; 98.5 Å³; Table 1). As with lactic acid, permeability for both physiological substrates increased toward lower pH, indicating passage of the neutral acids via AQP9 (Fig. 5). However, correlation of the permeability rates to the available fractions of neutral, protonated substrate acid in the buffer (Fig. 5, dotted lines) uncovered major differences: Permeability of acetic acid directly corresponded to the available neutral acid fraction, whereas with β-hydroxybutyric acid, availability and permeability curves were shifted by a full log unit, i.e. β-hydroxybutyric acid passed AQP9 10 times better than predicted from the free substrate concentration.

Discussion

Our results indicate that the protein surface charge of AQPs plays a critical role in the attraction and repulsion of weak monoacids. The negatively charged protein surfaces of murine AQP7 (Fig. 3A) and AQP9 (Fig. 3B) may be directly attributable to repulsion of weak monoacids by the negative surface electrostatics.

Figure 3. AQP surface charge. A, Poisson-Boltzmann surface electrostatics of tetrmeric wild-type and mutant human AQP9 and murine AQP7. The side-view cartoon of a AQP9 protomer (top center panel) depicts the position of the surface arginines (Arg^{51} and Arg^{53}) and histidine (His^{146}). B, section of a protein alignment of murine AQP7 (Mus musculus), human AQP9 (Homo sapiens), and L-lactic acid conducting AQPs: A. thaliana NIP2;1, L. plantarum GlpF1 and 4, and S. mansoni AQP. Shown is the extracellular loop A between transmembrane spans 1 and 2. Charged residues are highlighted in red (acidic) and blue (basic). The AQP9 Arg^{51}/Arg^{53} positions are labeled by triangles. The alignment was set using TEXshade (34).

Figure 4. Expression and permeability of AQP9 mutants with altered surface electrostatics. A, Western blot (75 μg of total protein/lane) showing expression of AQP wild-type and mutants as detected via an engineered N-terminal hemagglutinin epitope tag (total of 39 kDa). The additional bands at 78 kDa and below and above 120 kDa correspond to the partially SDS-resistant AQP dimers, trimers, and tetramers. B, glycerol permeability determined at pH 7 by light scattering. C and D, pH profiles of L-lactate/lactic acid permeability via AQP9-H146A (C) and AQP9-R51E,E53E (D). The inset in D shows pH-independent glycerol permeability of AQP9-R51E,E53E. For comparison, the position of the curve of wild-type AQP9 is shown by the dashed line. The error bars denote S.E. from three independent experiments.

Figure 5. AQP9 pH profiles for acetic (A) and β-hydroxybutyric acid permeability (B). The pH-dependent fractions of protonated, neutral acid and β-hydroxybutyric acid (β-HB) are indicated by the dotted lines. Note the different scales of the ordinates. All experiments were done in triplicate; the error bars denote S.E.
AQP7 and AQP9-R51E,R53E effectively prevent lactate/lactic acid from approaching the pore, resulting in low permeability. This negative electrostatic shield at the surface (repelling anions) combined with the positive field generated by the NPA and ar/R constrictions (excluding cations) can be seen as a so-far undescribed, double-layered selection mechanism of AQPs against charged molecules. It is discussed, however, that a negative protein surface attracts ammonium cations, NH4+, as a feature of AQPs that conduct ammonia, NH3 (35). In turn, a positively charged protein surface, as shown in this study on human AQP9, increases the local acid anion concentration promoting protonation and passage of the neutralized monoacid species via the AQP.

The extent of an electrostatic field is defined by the charge and the dielectric properties of the environment (36, 37). In a physiological setting with water as the universal solvent, i.e. a high dielectric constant, ε, of 78, the outreach of the field is small. Therefore, to act on a long range, the attracting protein surface must be highly charged. In AQP9, this is accomplished by eight arginine residues that are concentrated at the central interface of the AQP tetramer. By sequence comparison we found that other AQPs with reported lactic acid permeability carry positive residues at the respective positions, whereas in murine AQP7 a glutamate results in negative electrostatics (Fig. 3B). To date, there are few examples of AQPs with apparent monoacid conductance. This may be because the structural requirements are rarely met in the AQP family. Therefore, the sporadic cases of positive charge accumulation in the center of the tetramer surface is likely to be physiologically meaningful, e.g. for lactic acid permeability of the Lactobacillus GlpFs (16), or lactic and β-hydroxybutyric acid permeability of AQP9 in the human brain (17, 27, 28). In fact, in the physiological pH range of 5.5–7, AQP9 permeability of l-lactic acid was 5%, and that of β-hydroxybutyric acid was even 12% that of glycerol (see integral of ρsol over the physiological pH interval; Table 1).

Among the monoacid substrates, l-lactic and β-hydroxybutyric acid showed one-eighth and somewhat more than one-eighth that of glycerol (see integral of ρsol over the physiological pH interval; Table 1).

Mechanism of weak acid conduction by AQPs

The experimental procedures

Plasmid constructs, yeast culture, and Western blots

Human AQP9 (NCBI Gene ID 366) and mouse AQP7 (NCBI Gene ID 11832) were cloned via Spel and Xhol into the pDR196 vector (29). Site-directed mutagenesis was carried out by PCR using the QuikChange protocol (Agilent). For the AQP9-H146A mutant, the forward primer was 5′-GAAAATGCAA-CAGCAGCTATTTTTGCAAACATAC-3′. For the Arg51→Arg53 double mutants the forward primers were 5′-CAAGCTATTTCTCAGTXXXGGAXXTTTGGAGGGGTCACT-3′, where XXX = GCT for R51A,R53A and XXX = GAA for R51E,R53E. DNA mutations were confirmed by sequencing. Saccharomyces cerevisiae strain W303-1A jen1Δ ady2Δ (MATa; leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15) lacking endogenous lactate transporters was kindly provided by M. Casal. The yeast cells were transformed with pDR196 plasmids encoding AQP9, AQP9-H146A, AQP9-R51A,R53A, AQP9-R51E,R53E, or AQP7 or with the empty plasmid using the lithium acetate/single stranded carrier DNA/polylethylene- lycol procedure (41). Transformed yeast was grown at 29°C in selective medium without uracil and containing adenine, histidine, leucine, tryptophan, and 2% (w/v) glucose. All plasmid constructs carry an N-terminal HA epitope tag and a C-terminal 10× His tag. The HA tag was used for detection by Western blotting using a monoclonal mouse anti-HA antibody (1:5000; Roche) and a horseradish peroxidase-conjugated secondary
Yeast protoplast preparation and stopped‐flow light scattering assays

Cultured yeast cells were collected at an $A_{600}$ of 1 in mid‐logarithmical phase (2000 $\times$ g at 4 °C), washed, and incubated for 15 min in 2 ml MOPS buffer (50 mm, pH 7.2, 0.2% 2‐mercaptoethanol). For protoplastation, per gram of wet yeast 4 ml of MOPS/2‐mercaptoethanol buffer containing 1.8 M sucrose, 200 units of zymolyase‐20 T (MP Biomedicals, Illkirch, France), and 100 mg of bovine serum albumin fraction V (Roth, Karlsruhe, Germany) were added. The cell suspension was agitated for 60 min at 29 °C by orbital shaking at 100 rpm. Protoplasts were collected (2000 $\times$ g, 4 °C), washed, and resuspended in 2 ml of buffer (pH 7: 20 mM MOPS; pH 6: MES; pH 3, 4, and 5: citric acid, containing 1.2 M sucrose, 50 mM NaCl, and 5 mM CaCl$_2$). The protoplast suspensions were diluted to an $A_{600}$ of 2 before the assay. The measurements were done at 20 °C in a stopped‐flow apparatus (SFM‐300; BioLogic, Clai, France) with a dead time of ~5 ms, a total flow rate of 7 ml s$^{-1}$, and a total injection volume of 150 μl. Hypertonic permeability assays were carried out by rapidly mixing the protoplasts with the same volume of buffer (described above) supplemented with 100 mM solute (glycerol, acetate, L‐lactate, β‐D/‐hydroxybutyrate), generating a 50 mM inward gradient. For measuring the effect of protonophores at pH 4, 1 mM dinitrophenol was added 30 min prior to the assay. Protoplast volume changes were monitored by measuring the intensity of 90° light scattering at 546 nm. In each experiment 6–9 traces were averaged. The permeability coefficients ($P_{sol}$, μm s$^{-1}$) were obtained by exponential fitting (Biokine software) of the second phase of the light scattering curves using $P_{sol} = \frac{\left[dI/dt\right] \cdot (V_o \cdot C_{out})}{(S_o \cdot C_{diff})}$, with $dI/dt$ being the slope of the intensity curve, $V_o$ and $S_o$ are as above, $C_{out}$ is the total external solute concentration (1.25 M for glycerol; 1 M for monocarboxylates), and $C_{diff}$ is the chemical solute gradient (0.05 M). Three biological replicates were done for each experimental condition, and the S.E. was calculated.

Media alkalization measured by pH electrode

200‐ml yeast cultures were grown overnight to an $A_{600}$ of 1 and harvested (4000 $\times$ g, 5 min), washed, and resuspended in 2 ml of 4 mM citric acid buffer, pH 5, containing 0.5% 6‐deoxyglucose. The cells were resuspended to an $A_{600}$ of 60. 1 ml of cell suspension plus 7 ml of water were put on a magnetic stirrer, and pH changes were monitored with a glass membrane electrode after addition of 20 mM L‐lactate. In each experiment, three pH curves were averaged, and the resulting time constants were determined by linear fitting using SigmaPlot. Three biological replicates were done.

Electrostatics calculations

AQP9 and AQP7 protein structure models were generated based on E. coli GlcF using SwissModel (33) and displayed with the Chimera software (42). Linear Poisson‐Boltzmann electrostatics were calculated by calling the PDB2PQR (43, 44) and Adaptive Poisson‐Boltzmann Solver (APBS) (32) algorithms from within Chimera.

Mechanism of weak acid conduction by AQP8

Author contributions—M. R. conducted most of the experiments, analyzed the results, and wrote parts of the paper. D. R. started the project and carried out initial permeability measurements. E. B. conceived the idea for the project, ran the APBS electrostatics analyses, and wrote the paper with M. R.

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