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Review

Aquaporins and membrane diffusion of CO_2 in living organisms ☆

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ABSTRACT

Background: Determination of CO_2 diffusion rates in living cells revealed inconsistencies with existing models about the mechanisms of membrane gas transport. Mainly, these discrepancies exist in the determined CO_2 diffusion rates of bio-membranes, which were orders of magnitudes below those for pure lipid bilayers or theoretical considerations as well as in the observation that membrane insertion of specific aquaporins was rescuing high CO_2 transport rates. This effect was confirmed by functional aquaporin protein analysis in heterologous expression systems as well as in bacteria, plants and partly in mammals.
Scope of Review: This review summarizes the arguments in favor of and against aquaporin facilitated membrane diffusion of CO_2 and reports about its importance for the physiology of living organisms.
Major Conclusions: Most likely, the aquaporin tetramer forming an additional fifth pore is required for CO_2 diffusion facilitation. Aquaporin tetramer formation, membrane integration and disintegration could provide a mechanism for regulation of cellular CO_2 exchange. The physiological importance of aquaporin mediated CO_2 membrane diffusion could be shown for plants and cyanobacteria and partly for mammals.
General Significance: Taking the mentioned results into account, consequences for our current picture of cell membrane transport emerge. It appears that in some or many instances, membranes might not be as permeable as it was suggested by current bio-membrane models, opening an additional way of controlling the cellular influx or efflux of volatile substances like CO_2. This article is part of a Special Issue entitled Aquaporins.

1. The debate about membrane CO_2 diffusion and aquaporins

The gas exchange between cells and between cells and atmosphere is one of the preconditions for an intact physiology in almost all living organisms. For the majority of organisms, oxygen and CO_2 are the most significant gases with this regard. While in animals, plants, fungi and many bacteria oxygen is required as an electron acceptor for respiration, CO_2 is the product of catalytic reactions and exchanged with the atmosphere. In plants, CO_2 is also the substrate for sugar synthesis initiated and catalyzed during the reductive pentose-phosphate cycle, also known as the Calvin–Benson cycle. For this process, CO_2 diffuses in the opposite direction from atmosphere into the cells respectively organelles. Even though the diffusion and exchange of both gases might be of equal importance, this review will focus on the CO_2 diffusion and a possible involvement of aquaporins in that process due to the fact that there are currently more experimental data available to this regard. Despite this availability of information, the experimental data obtained for the diffusion of CO_2 through biomembranes are inconsistent even contradictory. Accordingly, the experts are split into one faction stating that CO_2 diffusion through membranes is so rapid that any membrane protein would reduce the flow. Consequently, a protein facilitating CO_2 membrane diffusion is not necessary and not existing. Measurements indicating that CO_2 membrane fluxes increase if certain proteins inserted were suspected to be incorrect because data was provided from experiments with possible technical problems. For example, if gas diffusion was monitored by a device such as a stopped flow spectrophotometer with a detection lag time larger than a theoretical calculated value for the diffusion time, the figures must reflect an artifact because the gas has diffused before the measurement begins. Nonetheless, scientists from the other faction claim that the measured CO_2 diffusion rates were by orders of magnitudes lower than the theoretical ones. If this holds true, proteins facilitating gas respectively CO_2 transport are mandatory for high diffusion rates and these in turn are one of the preconditions for smoothly running physiology processes. In this essay, we try to depict the arguments of both sides. However, results from our own studies revealed lower CO_2 permeability of biomembranes than estimated from theoretical considerations and a significant role of aquaporins in the diffusion of CO_2. Regarding this, it is clear that the authors favor the point of view of the latter faction.

2. Reasons for and against

Support for a significant role of aquaporins in gas diffusion came from the analysis of aquaporin function in heterologous expression systems [1–4]. Aquaporin membrane-insertion increased the cellular

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CO_2 uptake rates several fold. In the analysis of aquaporin antisense, RNAi and T-DNA knockout plants revealed a reduced CO_2 conductance [1,5–7]. It is generally acknowledged that plant science brought up the first evidence for the physiological function of an aquaporin as a CO_2 membrane transport facilitator [8,9]. These findings were not confirmed by studies in aquaporin knockout animals under physiologically relevant conditions [10,11]. This and the above mentioned objections from theoretical considerations caused a discussion about the mechanism of membrane volatile diffusion in general [12–14]. Important facts contributing to this discussion came from the pioneering work of Meyer and Overton [15,16] saying that the permeability coefficient of a solute is related to its oil–water partition coefficient. The "solubility–diffusion" model [17], which was developed on the basis of Meyer–Overton's work, predicts that the easier it is for a chemical to dissolve in a lipid, the easier and faster it will be transported into a cell. Consequently, lipophilic CO_2 could freely permeate across cell membranes and the diffusion rate-limiting step is independent from membrane characteristics but relies on unstirred water layers on both sides of the membrane [18]. To conflict sharply with this notion, some studies in animals, plants and heterologous expression systems came to the conclusion that a change in membrane characteristics was causing the observed effects [2,4,5,19–21]. The membrane CO_2 permeability was found to be 10 to 1000 times lower than that obtained for pure lipid bilayers [2,6,10,22–25]. The CO_2 transport rates were not limited by unstirred layer effects as predicted by the above-mentioned models but by membrane resistances towards CO_2 diffusion. Under these conditions, aquaporin-facilitated CO_2 diffusion could be a possibility to ensure high cellular CO_2 transport rates and, by this, the survival of the respective cell. Here, the question is, if it is justified to apply the Meyer–Overton correlation. The interpretation of the results obtained by Meyer–Overton was later on also challenged by the fact that some biological membrane had an extremely low CO_2 permeability [4]. Experimental approaches using black lipid bilayers [26] might be inappropriate because they insufficiently reflect the situation in living organisms. In fact, current models concede that the consistency of bio-membranes can range from liquid to semi-crystalline [27]. Membranes are patchy and lipid regions vary in thickness and composition. Protein crowding and ectodomains limit exposure of lipid to the adjacent aqueous regions. Lipid exposure could be further reduced by the lipid–protein ratio [27,28]. In artificial lipid bilayers containing up to 70 mol% cholesterol the CO_2 permeability was found to be 2 orders of magnitude lower than that of those without cholesterol. Reconstitution of human AQP-1 into cholesterol-containing vesicles increased CO_2 permeability values. Insertion and exclusion of gas permeable aquaporin would thus result in a rapid variation of cellular CO_2 conductance [29].

3. Molecular mechanism of aquaporin facilitated CO_2 diffusion

Aquaporins consist of six membrane-spanning helical domains with N- and C-termini heading towards the cytosol. Conserved NPA motifs in loop B and E from opposite sides located inside the membrane form the water-conducting channel [30]. Monomers of aquaporin function as single water channel; however, aquaporins tend to form tetramers [31]. In general, membrane proteins tend to form oligomers not only for stabilization but also for functionality of the protein [32]. However, the reason why some aquaporins tend to form tetramer is still not clear [33]. Besides the functionality of a water-channel, a function as facilitator for membrane transport of glycerol or volatile substances like CO_2 or NH_3 was demonstrated [1,34,35].

AQP1 is an aquaporin that has been examined well with regard to its function in CO_2 transport. Atomic molecular simulation data based on AQP1 crystal structures showed that the AQP1-mediated CO_2 transport could be expected in membranes with low intrinsic CO_2 permeability [36]. Experimental data also gave clear evidence that AQP1 could facilitate CO_2 transport in *Xenopus laevis* oocytes [1,2]. Based on the simulation data, an indication that the CO_2 transportation through the membrane could be mediated by the central pore formed by the tetramer. Later on, experimental data based on the artificial tetramer of NtPIP1;2 and NtPIP2;1 further proved that tetramer formation is necessary for CO_2 transportation [25].

The debate of CO_2 passing through membrane came from experiments showing that certain lipids had high CO_2 permeability and the unstirred layers (ULs) contribute much more in CO_2 resistance than a biological membrane. However, the groups of Boron and Endeward found that oocyte membranes appear to be specifically tight to CO_2, and in these oocytes, aquaporin insertion dramatically increased the CO_2 permeability [14]. Criticism on the applied technique and data interpretation was concerning the method of measuring CO_2 transport and the contribution of ULs to the resistance against CO_2 diffusion in biological membranes [14]. Recently, a new method for monitoring CO_2 transport through a lipid bilayer using a micro pH electrode was developed. CO_2 transportation through a two-chamber system that was separated by a lipid bilayer resulted in an acidified region close to the membrane. A micro pH electrode attached to a micromanipulator device was used to monitor this acidification [18,37]. This tool offered a new instrument to study the CO_2 diffusion through membranes independent from time and just reliant on the amount of diffusing CO_2. In addition, using non-CO_2 permeable triblock-copolymer membranes it was possible to reduce the background diffusion that is quite significant in lipid bilayers to almost zero. In these quasi CO_2 tight membranes, representatives from both major types of plant aquaporins, PIP1;2 and PIP2;1, can facilitate CO_2 diffusion. In other systems such as yeast cells, the function of PIP2;1 as CO_2 diffusion facilitator was not detected. This might be due to the higher background diffusion rates of yeast membranes in comparison to those from triblock-copolymers [37].

4. Physiological importance of aquaporins for CO_2 membrane transport

In comparison to expression data and studies on basic characteristics of aquaporin function just a relatively small number of publications about the physiological relevance of aquaporin facilitated CO_2 membrane diffusion in intact organisms are available. The first evidence for aquaporin facilitated membrane diffusion of CO_2 came from research on the human aquaporin, HsAQP1. Analyses on human red blood cells in which this aquaporin is expressed [2] indicate that the aquaporin has a function as a CO_2 channel. Further research indicates that aquaporins are heterologously expressed in Xenopus oocytes. HsAQP1 expressed in these oocytes considerably increases the membrane CO_2 permeability [3]. While both studies show a correlation between expressed aquaporins and membrane CO_2 permeability, a clear physiological relevance could not be shown [11].

4.1. Mammals

Gas permeability of cell membranes (P_{CO2}) in mammals can vary considerably. The apical membranes of gastro-intestinal endothelia exhibit unusually low CO_2 permeability [24], which contradicts the common view of gas permeable membranes. Hence, it was concluded that a permeability barrier to gases in certain types of membranes exists. High bacteria generated CO_2 partial pressures can occur in the intestine [38,39] and these high CO_2 concentrations can induce a serious acid load for epithelial cells if the CO_2 molecules can freely permeate across the plasma membrane into the cytosol. From a physiological point of view, it appears reasonable if some membranes build up a gas barrier to protect the cells. By comparison, CO_2 diffusion rates in organs and tissues, which are important for gas exchange, for example lung microvascular endothelia and red blood cells [2], membrane CO_2 permeability was determined to be very high, presumably to enable quick gas exchange.

In 2006 Endeward and coworkers reported about putative CO_2 transporters in red blood cell membranes. They observed that P_{CO2} of

red cells deficient in human aquaporin 1 as well as in cells lacking the Rh-associated glycoprotein, another CO_2 transporting protein complex [13], were reduced to about 0.07 cm/s. This is about half of the P_{CO2} figure of normal red blood cells. Blocking the Rh/RhAG system in absence of AQP1 reduced the red cell CO_2 permeability by as much as 95% compared to control cells. The conclusion was that both proteins account for the high CO_2 permeability of red cell membranes and are responsible for at least 50% of the total CO_2 membrane transport [2,13].

An evidence for the relevance of CO_2 transporting aquaporins in the physiology of whole mammalian organisms is still lacking. Lung gas exchange has been studied *in vivo* in anesthetized mice that have been ventilated with 5% CO_2 in oxygen. No difference in CO_2 transport could be shown in WT mice compared to AQP1 null mice [10]. A possible explanation for that unanticipated result might be found by the following explanation: red blood cells travelling with the blood stream through the pulmonary capillaries deliver their CO_2 load to the alveolar lumen and take up O_2. Whether a gas exchange equilibrium can be achieved is dependent on the contact time of red blood cells to the pulmonary capillaries. A limitation of CO_2 release to the alveolar lumen caused by the lack of AQP1 should be most prominent when the contact time is short, i.e. when the cardiac output is elevated and the blood stream runs faster due to exercise. To analyze this, voluntary exercise of WT and AQP1-null mice was studied on activity wheels. The distance run by knockout mice was found to be reduced by about 40% compared to WT mice [8,40], which might be due to CO_2 transport problems eventually leading to respiratory blood acidosis. However, it is not yet entirely clear if the reduced exercise tolerance was caused by limited CO_2 offloading or by limited O_2 uptake. Expression studies by Echevarria et al. [41] as well as molecular dynamics simulations [42] support the hypothesis that AQP1 might also be involved in oxygen membrane transport.

4.2. Plants

The first clear descriptions of a physiological relevance of aquaporin facilitated membrane CO_2 transport came from the field of plant science. A contribution of aquaporins to CO_2 transport and photosynthesis in plants has been suggested in 2002 by Terashima and Ono upon aquaporin inhibitor studies performed in *Vicia faba* that showed a reduction of photosynthetic activity under the respective treatment [9]. NtAQP1, an aquaporin belonging to the PIP1 subfamily, was the first plant aquaporin that has been shown to be a CO_2 transport facilitator when heterologously expressed in *Xenopus* oocytes or yeast cells [1,25]. Reduction of NtAQP1 expression in tobacco plants resulted in a reduction of cellular CO_2 uptake, chloroplast CO_2 concentration and photosynthetic performance [1,6].

In plants, CO_2 has to pass at least three membranes and other barriers [22] to reach the chloroplast stroma, which is the site of CO_2 fixation. Here, availability of CO_2 is rate limiting for photosynthesis, if adequate supply of light energy is available and the substrates for the Calvin–Benson cycle are present in sufficient concentrations. Resistance to CO_2 diffusion through the leaf tissue limits CO_2 diffusion and restricts CO_2 concentration in the chloroplast. The limitation of leaf internal gas diffusion by membranes has been neglected for a long time. Recently, *Arabidopsis thaliana* T-DNA insertion lines with a knockout of AtPIP1;2 were analyzed with this respect and it could be demonstrated that AtPIP1;2 as a CO_2 transport facilitator in heterologous expression systems had a similar function *in vivo*. The insertion mutant lines showed a reduction in internal CO_2 conductance and as a consequence a reduction in photosynthetic performance [7]. Knocking out a PIP2 aquaporin (AtPIP2;3), which could be proven to be a true water channel, had no effect on CO_2 related processes. In contrast to the latter findings, Hanba and coworkers were able to show that heterologous expression of a PIP2 aquaporin from barley (HvPIP2;1) increases internal CO_2 conductance, CO_2 concentration in the chloroplast and photosynthesis rates [43].

4.3. Cyanobacteria

A moderate water permeability of the aquaporin SsAQPZ from *Synechococcus sp.* PCC7942 was demonstrated in the *Xenopus* oocyte expression system as well as after reconstitution into liposomes. Using yeast cells expressing SsAQPZ and carbonic anhydrase aquaporin mediated CO_2 uptake was measured via fluoresceine fluorescence quenching in response to intracellular acidification. Yeast cells expressing SsAQPZ showed about a threefold increased CO_2 permeability compared to control cells [44]. *In vivo* studies suggested a role for SsAQPZ in cellular CO_2 uptake. *Synechococcus sp.* PCC7942 wildtype cells grew faster in liquid culture than SsAQPZ knockout cells and also showed increased growth rates on solid medium. WT cells performed better than knockouts under a wide range of applied CO_2 concentrations.

5. Conclusions

Even though the debate about the mechanism of membrane CO_2 diffusion continues and it is difficult to draw general conclusions, recent findings indicate that the regularities for black lipid bilayer could not be applied to bio-membranes in or from living organisms in every case. A reduction of CO_2 diffusion rates by orders of magnitudes lower than theoretical levels as it was experimentally demonstrated justifies the role of certain aquaporins as CO_2 diffusion facilitators. Aquaporin-function would enable rapid gas exchange which allows efficient physiological reactions. Some references indicate that the aquaporin CO_2 conductivity resides on the so called central or 5th pore formed by the constitution of a tetramer. Assembly and disassembly of a tetramer would, at the same time, also change CO_2 diffusion rates and provide a mechanism of controlling or modifying CO_2 diffusion rates. Taking these data into account, consequences for our current picture of cell membrane transport emerge. It appears that in some or many instances, membranes might not be as permeable as it was suggested by current bio-membrane models opening an additional way of controlling the cellular influx or efflux of volatile substances like CO_2.

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