Reconstituted Aquaporin 1 Water Channels Transport CO₂ across Membranes*

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Biological membranes provide selective barriers to a number of molecules and gases. However, the factors that affect permeability to gases remain unclear because of the difficulty of accurately measuring gas movements. To determine the roles of lipid composition and the aquaporin 1 (AQP1) water channel in altering CO₂ flux across membranes, we developed a fluorometric assay to measure CO₂ entry into vesicles. Maximal CO₂ flux was ~1000-fold above control values with 0.5 mg/ml carbonic anhydrase. Unilamellar phospholipid vesicles of varying composition gave widely varying water permeabilities but similar CO₂ permeabilities at 25 °C. When AQP1 purified from human red blood cells was reconstituted into proteoliposomes, however, it increased water and CO₂ permeabilities markedly. Both increases were abolished with HgCl₂, and the mercurial under certain circumstances, gas permeation through the water pore; layer composition or fluidity. AQP1 clearly serves to facilitate CO₂ permeation through the lipid bilayer and diffusing through the hydrophobic region of the lipid molecules (1, 2). Until recently, small uncharged molecules, however, may traverse the lipid bilayer. Uncharged molecules, such as water, were believed to diffuse freely across the bilayer (1, 2). However, membranes in certain circumstances, gas permeation through membranes is protein-mediated.

Ions require specific membrane transporters or channels to traverse the lipid bilayer. Uncharged molecules, however, may cross the bilayer either through specific channels or by partitioning into the bilayer and diffusing through the hydrophobic region of the lipid molecules (1, 2). Until recently, small uncharged molecules, such as water, were believed to diffuse freely across the bilayer (1, 2). However, membranes in certain circumstances can restrict movement of small uncharged molecules as well (3), requiring the presence of selective channels like those for water (aquaporins) to permit the flux of these small nonelectrolytes (3, 4).

Membrane permeability to gases has been less extensively studied, however, because of the difficulty of accurately measuring gas fluxes. Gross movement of O₂ and CO₂ across the lung has been measured, as has NH₃ permeability of some membranes (5–7). Although it has been accepted that gases are freely permeable across membranes, rapid flux of gases across some membranes would upset carefully regulated electrolyte concentrations. For example, NH₃/NH₄⁺ balance requires restricted permeability to NH₃, and low NH₃ permeability has been demonstrated in gastric glands (7) and renal thick ascending limb epithelia (6, 8). Recent studies have also provided evidence that aquaporins may be capable of mediating flux of CO₂ across membranes (9).

We have developed a method to measure CO₂ fluxes across the membranes of unilamellar vesicles. In liposomes or proteoliposomes with entrapped 5,6-carboxyfluorescein (CF)¹ and carbonic anhydrase, we measured the rate of pH drop engendered when abrupt exposure to external CO₂/HCO₃⁻ leads to rapid entry of CO₂ into the vesicle and generation of carbonic acid. Using this method we compared water and CO₂ permeability of a variety of membranes and demonstrated that aquaporin 1 (AQP1) reconstituted as the sole protein in proteoliposomes mediates CO₂ flux.

MATERIALS AND METHODS

Vesicle Preparation—Powdered lipids were suspended in 50 mM NaCl, 50 mM KCl, 20 mM HEPES, pH 7.40, and either 1.0 mM CF for CO₂ measurements or 20.0 mM CF for water measurements (Molecular Probes, Inc., Eugene, OR), either without or with 0.5 mM bovine erythrocyte carbonic anhydrase (CA) (3240 Wilbur-Anderson units/mg of protein). Vesicles were prepared by heating to 40 °C and then cooling on ice three times, followed by 20 serial extrusions of the mixture through a 0.1-μm pore polycarbonate filter using an Avanti mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL) warmed on a heating block to ~50 °C as described (5, 10, 11). Before flux measurements, extravesicular CF was removed by passing the liposomes through a Sephadex G-50 column as described previously (11). Previous studies have demonstrated that this approach forms unilamellar vesicles (5, 12); median vesicular size was determined by quasienstactic light scattering (5, 11).

Flux Measurements—For CO₂ permeability (Pₑ), measurements, vesicles were abruptly exposed to a CO₂ gradient by rapidly mixing with an equal volume of freshly made 0.1 mM NaHCO₃ and 20 mM HEPES, pH 7.40, that was kept capped throughout the experiment. Fluorescence changes were measured on a stopped-flow device as described (5, 10–12). Fluorescence data from 8–10 individual determinations were averaged and fit to a single exponential curve. The buffer capacity of the vesicle interior was calculated from the fluorescence change in response to the addition of HCl, sodium acetate, and NaHCO₃ solutions as described (10, 13) and had a value of 26.9 mM/pH unit. The buffer capacity was then used to correlate the fluorescence change to the pH change, as described (10, 13). For water permeability (Pₑ) measurements, vesicles were identically prepared except 20.0 mM CF was used and the vesicles were abruptly exposed to hyperosmolar external solution. Sufficient sucrose was added to the external medium so that solution osmolality doubles when equal volumes of external medium and vesicles are mixed. Pₑ was measured and calculated as described (5, 10–13).

Preparation of AQP1 Proteoliposomes—Purified AQP1 was prepared from outdated human packed red blood cells exactly as described (12, 14–16). AQP1 was purified in solution containing 20 mM Tris-Cl, pH 7.80, containing 1.0 mM Na⁺, 1.0 mM dithiothreitol (DTT), and 1% (v/v) n-octyl glucoside (Calbiochem) before reconstitution. SDS-polyacrylamide gel electrophoresis and immunoblotting were performed by

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¹The abbreviations used are: CF, 5,6-carboxyfluorescein; AQP1, aquaporin 1; CA, carbonic anhydrase; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid.
standard techniques. Reconstitution into proteoliposomes was performed as described (12, 16). Briefly, 9 mg of bath-sonicated, purified Escherichia coli phospholipid (Avanti Polar Lipids, Inc.) was mixed with 1.0 ml of a solution containing 50 mM Tris-Cl, pH 7.50, 1.0 mM NaCl, 1.0 mM DTT, 1.25% (v/v) n-octyl glucoside, and 100 μg of purified AQP1. This mixture was vortexed, placed on ice for 20 min, and rapidly injected through a 25-gauge needle into 25 ml of reconstitution buffer (50 mM MOPS, pH 7.50, 15 mM CF, 1.0 mM DTT, and 0.5 mM phenylmethanesulfonyl fluoride) at room temperature. Proteoliposomes were collected by centrifugation at 123,000 g for 1 h at 4°C. Two to three washes were performed in CF-free reconstitution buffer. E. coli phospholipid liposomes without AQP1 were prepared similarly except that an equivalent volume of the control AQP1 was prepared similarly except that an equivalent volume of the capacity was 23.9 mM/pH unit.

to pH changes as described above except that the measured buffer was made in the absence of CA or in the presence of 0.5 mg/ml CA (inset). A CA titration curve was generated, and a concentration of 0.5 mg/ml was sufficient to evoke the maximal rate of pH change (data not shown).

RESULTS AND DISCUSSION

The uncatalyzed hydration of CO2 is slow, and the reaction is catalyzed in vitro by CA. When measuring CO2 flux across a bilayer in vitro, it is important to demonstrate that the flux is not rate-limited by the hydration reaction. Therefore, we measured the flux of CO2 across the bilayer of lipid vesicles in the absence and presence of entrapped CA. In the absence of CA, the hydration of CO2 is clearly rate-limiting because the addition of CA causes a 1000-fold increase in the rate of pH change (Fig. 1). Varying the amount of entrapped CA revealed no increase in the rate of acidification at levels above 0.2–0.3 mg/ml. Therefore, 0.5 mg/ml CA was included in all subsequent CO2 flux measurements.

We first determined the effect of lipid composition on the rate of CO2 entry into vesicles. We had previously shown that Pf of synthetic phospholipid vesicles varies directly with the fluidity of the bilayer (5). To determine whether membrane fluidity affects CO2 permeability in a similar manner, we measured the water and CO2 permeabilities of phospholipid vesicles that are known to represent a wide range of bilayer fluidities (5). Vesicles exhibited unimodal size distributions with an average diameter of 263 ± 78 nm (n = 42) (data not shown). As shown previously (5) water permeabilities for the three vesicle compositions varied by more than 130-fold (Fig. 2 and Table I), ranging from 0.208 ± 0.066 × 10⁻³ cm/s for the least fluid bilayer (60% sphingomyelin:40% cholesterol) to 28.0 ± 2.7 × 10⁻³ cm/s for the most fluid bilayer (dilinoleoyl lecithin). The CO2 permeabilities for lipid vesicles of the same compositions, however, did not vary significantly (Fig. 2 and Table I), with all three compositions having CO2 permeabilities of ~1.55 × 10⁻³ cm/s. Therefore, membrane fluidity does not govern the permeability of lipid vesicles to CO2.

Given that some membranes restrict water and gas fluxes (1–3, 6, 10, 17, 18) across the lipid bilayer, it is possible that specific channels are necessary to regulate the flow of these substances across biological membranes. Indeed, it is now well established that specific water channels (aquaporins) mediate water flow across membranes (3, 4). Furthermore, in principal cells of the collecting duct of the kidney, water flow is hormonally regulated by the insertion of AQa2-containing vesicles into the apical membrane (19). It has recently been shown that the AQP1 water channel causes a 40% increase in CO2 permeabil-

![Fig. 1. Effect of CA on the rate of CO2 movement across lipid bilayers. The pH change that occurs as CO2 enters and acidifies the interior of the vesicles is shown as a function of time. The exponential fit used to calculate the rate for the data is shown by the smooth line. Measurements were made in the absence of CA or in the presence of 0.5 mg/ml CA (inset). A CA titration curve was generated, and a concentration of 0.5 mg/ml was sufficient to evoke the maximal rate of pH change (data not shown).](http://www.jbc.org/)

![Fig. 2. Effect of membrane fluidity on water and CO2 permeability. Either the change in vesicular volume (a measure of Pf) (top) or the change in internal pH (a measure of CO2 permeability) (bottom) was measured as a function of time. Lipid compositions (all mol/mol) were chosen to represent a range of water permeabilities with vesicles composed of 60% sphingomyelin:40% cholesterol (Sph:Chl) representing low fluidity composition, 80% 1-palmitoyl-2-oleoyl lecithin:20% cholesterol (POPC:Chl) representing intermediate fluidity composition, and 100% dilinoleoyl lecithin (DLPC) representing high fluidity composition. Addition of 0.5 mg/ml CA to water permeability experiments had no effect (data not shown).](http://www.jbc.org/)

| Table I Water and CO2 permeabilities of liposomes and proteoliposomes |
|------------------|------------------|------------------|
| Lipid composition | Water Pf | CO2 Pco2max |
|------------------|------------------|------------------|
| 60% sphingomyelin/40% cholesterol | 0.208 ± 0.066 | 1.34 ± 0.63 |
| 80% 1-palmitoyl-2-oleoyl lecithin/20% cholesterol | 12.6 ± 4.6 | 16.8 ± 0.72 |
| 100% dilinoleoyl lecithin | 28.0 ± 2.7 | 1.54 ± 0.13 |
| E. coli phospholipid | 4.61 ± 4.04 | 0.53 ± 0.14 |
| AQP1 reconstituted into E. coli phospholipid | 18.4 ± 5.8 | 1.94 ± 0.72 |
ity when expressed in Xenopus oocytes (9). However, the authors were unable to distinguish among three explanations for this increase: 1) a change in lipid composition of the cell membrane, 2) increased expression of a native gas channel, or 3) direct mediation of gas transport by AQP1. Our results argue against a large effect of lipid composition, so we addressed the questions of other gas channels and AQP1 permeability directly by purifying and reconstituting AQP1 into proteoliposomes and measuring the CO₂ permeability.

As reported previously, AQP1 purified from red blood cells was purified to homogeneity and represented the sole protein in proteoliposome preparations (12, 16). Fig. 3A shows the water permeabilities for liposomes containing no AQP1 and for proteoliposomes containing the protein. Proteoliposomes containing AQP1 exhibited a 4-fold higher $P_f$ than liposomes lacking the protein (upper panel). AQP1 contains a cysteine residue at position 189 that is essential for function and that is sensitive to mercurial compounds (20). In the lower panel of Fig. 3A, the effects of blocking this cysteine with HgCl₂ and of reversing the blockade with β-mercaptoethanol are shown. HgCl₂ reduced $P_f$ to values similar to those of liposomes lacking AQP1, and mercaptoethanol reversed this effect.

We next examined the effect on CO₂ permeability of reconstituting AQP1 into proteoliposomes (Fig. 3B). As occurred with $P_f$, addition of AQP1 also results in an approximately 4-fold increase in CO₂ permeability (upper panel), the liposomes having a permeability of $0.53 \pm 0.14$ cm/s and AQP1 proteoliposomes exhibiting a permeability of $1.94 \pm 0.72$ cm/s. The increase in CO₂ permeability that results from incorporation of AQP1 is again eliminated by HgCl₂, and the inhibition by HgCl₂ is again reversed by β-mercaptoethanol. This demonstrates that the AQP1 protein can serve as a CO₂ channel. These results further suggest that water and CO₂ may traverse the same pathway because the permeabilities both increase by approximately 4-fold and because both permeabilities exhibit the same reversible inhibition by HgCl₂. Because the AQP1 conductance for water is known (16), the conductance of AQP1 for CO₂ can be calculated. Under the conditions of the present studies, the AQP1 conductance for CO₂ is $2.0 \times 10^{-16}$ mmol of CO₂ s⁻¹ AQP1⁻¹ molecule. The water and CO₂ permeabilities of liposomes and proteoliposomes are also summarized in Table I.

We have clearly demonstrated that AQP1 can increase CO₂ permeation of lipid bilayers, likely through the water pore of the protein. The physiological relevance of this finding is not certain, but the abundance of AQP1 in tissues that serve to transport CO₂ such as red blood cells and in tissues that are intimately linked to bicarbonate-based pH control such as blood cells (21), renal proximal tubule (4), and choroid plexus (22) suggests that there may be an evolutionary advantage to rapid equilibration of CO₂ across these membranes. Studies of the permeabilities of other gases and studies in AQP1-deleted animals will provide further information about the physiological basis for this phenomenon.

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as a function of time, and the exponential fit is shown. The upper panels show the permeability of reconstituted AQP1 compared with identical lipid vesicles lacking AQP1. The bottom panels show that the permeability of AQP1 to both water CO₂ can be inhibited by 1 mM HgCl₂ and that this inhibition can be reversed with the addition of 5 mM β-mercaptoethanol (βME).

Fig. 3. Water and CO₂ permeabilities of AQP1. Either the change in vesicular volume (A) or the change in internal pH (B) was measured...
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