Exploring the Potential Roles of Band 3 and Aquaporin-1 in Blood CO₂ Transport–Inspired by Comparative Studies of Glycophorin B-A-B Hybrid Protein GP.Mur

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The Cl⁻/HCO₃⁻ exchanger band 3 is functionally relevant to blood CO₂ transport. Band 3 is the most abundant membrane protein in human red blood cells (RBCs). Our understanding of its physiological functions mainly came from clinical cases associated with band 3 mutations. Severe reduction in band 3 expression affects blood HCO₃⁻/CO₂ metabolism. What could happen physiologically if band 3 expression is elevated instead? In some areas of Southeast Asia, about 1–10% of the populations express GP.Mur, a glycophorin B-A-B hybrid membrane protein important in the field of transfusion medicine. GP.Mur functions to promote band 3 expression, and GP.Mur red cells can be deemed as a naturally occurred model for higher band 3 expression. This review first compares the functional consequences of band 3 at different levels, and suggests a critical role of band 3 in postnatal CO₂ respiration. The second part of the review explores the transport of water, which is the other substrate for intra-erythrocytic CO₂/HCO₃⁻ conversion (an essential step in blood CO₂ transport). Despite that water is considered unlimited physiologically, it is unclear whether water channel aquaporin-1 (AQP1) abundantly expressed in RBCs is functionally involved in CO₂ transport. Research in this area is complicated by the fact that the H₂O/O₂-transporting function of AQP1 is replaceable by other erythrocyte channels/transporters (e.g., UT-B/GLUT1 for H₂O; RhAG for CO₂). Recently, using carbonic anhydrase II (CAII)-filled erythrocyte vesicles, AQP1 has been demonstrated to transport water for the CAII-mediated reaction, CO₂(g) + H₂O ⇌ HCO₃⁻(aq) + H⁺(aq). AQP1 is structurally associated with some population of band 3 complexes on the erythrocyte membrane in an osmotically responsive fashion. The current findings reveal transient interaction among components within the band 3-central, CO₂-transport metabolon (AQP1, band 3, CAII and deoxygenated hemoglobin). Their dynamic interaction is envisioned to facilitate blood CO₂ respiration, in the presence of constantly changing osmotic and hemodynamic stresses during circulation.

Keywords: band 3, aquaporin-1, CO₂ transport, erythrocytes, metabolon, glycophorin, GP.Mur
INTRODUCTION

Band 3 (SLC4A1), a membrane protein of 911 amino acids, belongs to the SLC4A family of HCO$_3^-$ (and CO$_2^-$) transporters (Choi, 2012; Romero et al., 2013). Band 3 is also known as anion exchanger (AE1), as band 3-mediated HCO$_3^-$ transport is coupled at a 1:1 ratio with an anti-parALLEL flux of Cl$^-$, the most abundant anion physiologically. A truncated isoform of AE1, which lacks the first 65 amino acids of band 3 or erythroid AE1, is expressed in the acid-secreting intercalated cells of the kidney (Kollert-Jons et al., 1993). Band 3 is the most abundant membrane protein in human erythrocytes (1–1.2 million molecules per RBC), with two major functions: (1) cell mechanical support through its physical linkage to ankyrin and the cytoskeletal network (Low et al., 1991); (2) blood CO$_2$/HCO$_3^-$ exchange through its bicarbonate transport activity. The latter impacts acid-base homeostasis in various human physiological systems. This review first explores the function of band 3 in blood CO$_2$ exchange based on previous studies using cellular/animal models with different band 3 levels. In my laboratory, this was achieved experimentally by comparing RBCs expressing an unusual surface antigen named GP.Mur, to RBCs devoid of this antigen. GP.Mur RBCs generally express 20% or more band 3, and can be used as an experimental model for higher band 3 expression in erythrocytes (Hsu et al., 2009, 2011).

CLUES FROM EARLY GP.Mur PROTEOMIC RESEARCH

GP.Mur, commonly known as Miltenberger subtype III (Mi.III) in Southeast Asia, is an erythrocyte antigen of the MNS blood group system (Tippett et al., 1992; Lomas-Francis, 2011). The prevalence of GP.Mur is between 1 and 7% in regions of Southeast Asia including Taiwan, but very low in other parts of the world (Issitt, 1985; Broadberry and Lin, 1994; Hsu et al., 2013). GP.Mur structurally exhibits the configuration of glycophorin B-A-B (Figure 1). GP.Mur evolved from homologous gene recombination of glycophorin B and glycophorin A, and is essentially glycophorin B with a piece of glycophorin A inserted in the middle (Figure 1) (Huang and Blumenfeld, 1991; Hsu et al., 2015b). Antibodies against the antigenic Mur sequence at one of the two cross-over sites (e.g., anti-Mur; anti-Mi$^a$) are naturally occurring in ~0.5% local Taiwanese. So if a person bearing such an alloantibody is accidentally transfused with GP.Mur RBCs, an acute intravascular hemolytic reaction might occur (Lin and Broadberry, 1998). There are ~1 x 10$^6$ glycophorin A (GPA) protein molecules and 1.7–2.5 x 10$^5$ glycophorin B (GBP) molecules in an average human RBC (Gardner et al., 1989). In people with heterozygous GYP.Mur (GYP.Mur$^{het}$), GP.Mur replaces half of GBP protein expression; in people with homozygous GYP.Mur (GYP.Mur$^{hom}$), GP.Mur substitutes all the expression of GBP.

The early proteomic work from my group identifies unique structural features of GP.Mur-associated protein complexes on the RBC membrane (“ghosts”). Importantly, GP.Mur red cells express significantly more band 3. The protein-protein interaction between band 3 and AQPI on the GP.Mur RBC membrane is substantial, compared to that on the GP.Mur-negative cell membrane (Hsu et al., 2009). Band 3 and AQPI were thought not to interact on the erythrocyte membrane (Cho et al., 1999).

Because the only known difference between GP.Mur and GP.Mur-negative erythrocytes is the inserted sequence from glycophorin A in GYP.Mur (GYP.B-A-B), we hypothesized that the glycophorin A-derived peptide in GP.Mur promoted the protein expression of band 3. We took the experimental approach similar to an early study done in Xenopus oocytes, which demonstrates increase of band 3 levels upon GPA co-expression (Groves and Tanner, 1992). We transfected band 3 alone, or together with GP.Mur (or GPA as the positive control) into HEK-293 mammalian cultured cells. We found that like GPA, GP.Mur also enhances band 3 co-expression in the heterologous expression system (Hsu et al., 2009). For comparison, GPB appears to lack the chaperone-like activity of GPA for band 3, and is unable to promote band 3 protein expression (Groves and Tanner, 1992, 1994). Thus, the 32-aminio acid-long peptide inserted in GP.Mur, which GBP lacks, renders GP.Mur to be functionally equivalent to GPA in supporting band 3 expression (Figure 1) (Hsu et al., 2009).

The interaction between GPA and band 3 begins in the endoplasmic reticulum (ER) where GPA facilitates protein synthesis of band 3 (Groves and Tanner, 1992). GPA-band 3 complexes are found on the cell surface, but the two proteins also traffic independently to the plasma membrane (Pang and Reithmeier, 2009; Giger et al., 2016). This may explain why overall band 3 expression does not seem to be substantially affected by GPA-deficiency in individuals with the very rare blood types that lack GPA expression, i.e., En(a-), M$^b$M$^k$, and Mi.V (Bruce et al., 2004). On the other hand, in the RBCs with the rare En(a-), M$^b$M$^k$, and Mi.V phenotypes, the absence of GPA prolongs the retention time of band 3 in the ER and Golgi apparatus, resulting in excessive build-up of N-glycans in band 3 (Gahnberg et al., 1976; Bruce et al., 2004). Therefore, band 3 biosynthesis is slower without the chaperone-like activity of GPA. These structural features of GPA-deficient band 3 complexes are associated with their lower efficiencies in anion transport (e.g., for Cl$^-$, I$^-$, and sulfate$^-$) (Bruce et al., 2004). By fluorescence polarization studies, GPA-deficient band 3 complexes on the erythrocyte membrane also exhibit higher degrees of rotational freedom (Bruce et al., 2004), though a much earlier measurement shows that GPA or its substantial sialic acid content does not drastically affect the rotational freedom of band 3 (Nigg et al., 1980). The discrepancy was recently rigorously explored by single particle tracking of separately labeled band 3 and GPA. That new work reveals that GPA-band 3 interaction could become transitory during physiological processes like band 3 phosphorylation (Giger et al., 2016).
FIGURE 1 | Glycophorin B-A-B hybrid protein GP.Mur interacts with band 3. (Top) Protein sequence alignment of GPA, GPB, and GP.Mur, which was first published in Blood (Hsu et al., 2009), is modified here with color coding. The sequences of GPA and GPB are color-coded with blue and pink, respectively. The antigenic Mur peptide at the cross-over region in GP.Mur is color-coded purple. The configuration of glycophorin B-A-B is revealed by the combination of pink-(purple)-blue-pink colors. (Bottom) GPA, GPB, and GP.Mur are homologous membrane proteins, each with a single transmembrane span. Their N-terminal sequences are located extracellular, with heavy glycosylation (not shown); their C-terminal sequences are intracellular. Both GPA and GP.Mur interact with band 3 (gray cylinder), and GPB does not.

BAND 3 AS THE RATE-LIMITING FACTOR FOR BLOOD CO₂ RESPIRATION

In human, over two-thirds of CO₂ metabolite from tissues are carried in the form of soluble bicarbonate until exhalation in the lungs. By utilizing CO₂(g)/HCO₃⁻(aq) exchange, the capacity of one's CO₂ tolerance can be expanded substantially. This chemical conversion, CO₂(g) + H₂O ⇌ HCO₃⁻(aq) + H⁺(aq), is primarily facilitated by intraerythrocytic carbonic anhydrase II (CAII), as the rate of its spontaneous conversion outside RBCs (t½ = 14 s) is too slow to meet physiological demands (Reithmeier, 2001).

When blood circulates to the capillary bed, CO₂ metabolite enters RBCs via diffusion and/or gas channels (e.g., AQP1 and RhAG) (Cooper and Boron, 1998; Nakhoul et al., 1998; Endeward et al., 2008; Musa-Aziz et al., 2009). Abundant CAII inside erythrocytes (10⁶ molecules/RBC) facilitates hydration of CO₂(g) to HCO₃⁻(aq). Bicarbonate permeates through band 3 dimers or dimerized dimers (tetramers), following its concentration gradient across the RBC membrane (Reithmeier et al., 2016). Because HCO₃⁻ transport by band 3 utilizes Cl⁻ as the counter anion, band 3-mediated bicarbonate flux is electroneutral, and consumes very little or no energy. In the lung alveoli, CO₂ expiration drives the above reaction toward the left (dehydration of bicarbonate). Extracellular bicarbonate needs to enter red cells via band 3 to be converted into CO₂(g) by intraerythrocytic CAII (Figure 2, top).

CAII and band 3 are structurally and functionally coupled during blood CO₂ transport and respiration (Sowah and Casey, 2011). Carbonic anhydrase is the most efficient enzyme known today, with K_cat or its turnover number (~6 × 10⁵/sec) at least 10 times faster than the rate of Cl⁻/HCO₃⁻ exchange of an erythroid AE1 molecule (~5 × 10⁴ ions/sec) (Maren, 1967; Brahm, 1977). Since each human red cell expresses identical numbers of CAII and band 3 molecules (each ~10⁶ molecules/RBC), the efficiency of CAII-catalyzed CO₂/HCO₃⁻ conversion is about an order higher than the rate of AE1-conducted Cl⁻/HCO₃⁻ flux through the cell membrane. For comparison, the rate of water permeation through an AQP1 is ~3 × 10⁹ H₂O/sec. Intraerythrocytic water is used in CO₂/HCO₃⁻ exchange. With 160,000–200,000 AQP1 on the erythrocyte membrane, the rate of water transport via AQP1 is estimated 480–600 times faster than the enzymatic
activity of intraerythrocytic CAII. Therefore, the anion exchange activity of erythroid AE1 is the rate-limiting step for blood CO₂ transport (Reithmeier, 2001), which is directly related to the capacity of CO₂ respiration in an individual (Hsu et al., 2015a).

THE FUNCTION OF BAND 3 IN RESPIRATORY PHYSIOLOGY

Low or No Band 3 Expression

The respiratory support by erythroid AE1 is critical for human survival (Daniels, 2013). Up to date, there were only three clinical cases about the complete absence of band 3 protein expression resulted from deleterious homozygous mutations of band 3 (Ribeiro et al., 2000; Picard et al., 2014; Kager et al., 2015). These three patients between 3 and 4 years old at the time of publishing the case reports survive essentially by relying on regular transfusion. They were all delivered prematurely, because of fetal hydrops and severe anemia. One of them, later identified to be the only case of Southeast Asian Ovalocytosis (SAO) band 3 homozygosity in the world, was initially rescued by in utero transfusion at 22 weeks gestation, and then delivered at 29 weeks gestation (Picard et al., 2014). Intriguingly, besides apparent acidosis due to the absence of Cl⁻/HCO₃⁻ transport, bone marrow analyses for the three patients revealed similarly marked dyserythropoiesis. In the homozygous SAO patient, his dyserythropoiesis is characterized by abnormally larger erythroblasts with two or more nuclei (Picard et al., 2014), which resonates the main features of band 3 knockout in zebrafish—erythroid-specific cytokinesis and dyserythropoietic...
anemia (Paw et al., 2003). Therefore, in addition to blood CO\(_2\) transport, band 3 is functionally essential for correct mitosis during erythropoiesis.

SAO is prevalent in certain ethnic groups in Southeast Asia and the Southwest Pacific (Kimura et al., 2003). Besides one case of SAO homozygosity survived by rigorous medical intervention as mentioned above (Picard et al., 2014), almost all SAO cases are resulted from heterozygous mutation of SAO band 3 (Daniels, 2013). SAO mutation is characterized by gene deletion in codons 400–408 of band 3 that corresponds to a location at the border between the cytoplasmic domain and the first transmembrane domain (Liu et al., 1990; Jarolim et al., 1991). The red cells from heterozygous SAO carriers express heterodimeric band 3 that is composed of a SAO band 3 subunit and a normal band 3 subunit. From transport kinetic studies, the anion transport efficiency of SAO heterozygotes is only half of the transport efficiency of normal cells (Jennings and Gosselink, 1995). Heterozygous SAO patients presumably survive with ~50% efficiency of physiologic Cl\(^-\)/HCO\(_3^-\) exchange. For SAO patients comorbid with distal renal tubular acidosis (dRTA)-associated mutations in band 3, their band 3-mediated HCO\(_3^-\) transport is further reduced to less than 5% of the transport efficiencies (Jarolim et al., 1991; Liu et al., 1994; Bruce et al., 2000).

Intriguingly, total deficiency of band 3 has been found in a breed of Japanese black cattle with a premature stop codon in the coding sequence of band 3, which corresponds to codon 646 in human band 3 (Inaba et al., 1996). Despite that few of these band 3\(^{-/-}\) cattle survive to adulthood, the survived ones present growth retardation, severe anemia, and pathophysiological conditions related to band 3 deficiencies, i.e., membrane instability-related spherocytosis, reduced Cl\(^-\)/HCO\(_3^-\) anion fluxes across the erythrocyte membrane, mild acidosis, and smaller capacities for blood CO\(_2\) transport (Inaba et al., 1996).

These phenotypes of band 3-deficient cattle could be recapitulated in the above-mentioned, transfusion-dependent human cases and in the neonates of band 3\(^{-/-}\) mice: growth retardation, spherocytosis, severe anemia, and substantially reduced capacities of anion transport by RBCs (Inaba et al., 1996; Peters et al., 1996; Southgate et al., 1996; Ribeiro et al., 2000; Picard et al., 2014; Kager et al., 2015). But unlike the naturally bred, band 3-deficient cattle found in Japan, band 3 knockout mice do not survive for more than 2 weeks postpartum. The proportion of the homozygous neonates generated was about one quarter, obeying the Mendel's Laws of Inheritance (Southgate et al., 1996). Therefore, complete deficiency of band 3 does not affect the survival of band 3\(^{-/-}\) mice in the fetal stage, but postnatally. For human fetuses, blood CO\(_2\) transport primarily relies on CO\(_2\) and not HCO\(_3^-\), due to restricted bicarbonate permeation through the placenta (Gabbe, 2012). Inferred from the findings in human, cattle and mouse, HCO\(_3^-\) transporter band 3 becomes functionally critical for CO\(_2\) respiration after birth.

**High Band 3 Expression**

On the other end of the spectrum of band 3 expression is GP.Mur. GP.Mur erythrocytes express significantly more band 3, and present phenotypes opposite to the phenotypes from band 3-deficient RBCs, i.e., superior membrane resilience toward stress and larger capacities for CO\(_2\) transport and pH buffering (Inaba et al., 1996; Peters et al., 1996; Southgate et al., 1996; Hsu et al., 2009). These functional advantages of GP.Mur RBCs due to high band 3 levels are expected. But could this explain the long-standing observation of superior physical endurance and athleticism prevalent in Taiwanese from the tribes with exceedingly high frequencies of GP.Mur phenotype (Hsu et al., 2009; Hsu, 2011)? To probe into the impacts of GP.Mur (or higher band 3 expression) at the systemic level, we conducted a large human study on respiratory physiology. We challenged the recruited healthy adults with “3-minute stepping exercise” (a standardized fitness test), which temporarily increased the demands for respiratory gas exchange. Respiratory parameters were measured before and right after the exercise challenge. Indeed, people with GP.Mur blood type breathed and exhaled CO\(_2\) significantly faster than those lacking this blood type (about 1 minute faster in clearance of CO\(_2\) generated from 3-minute exercise) (Hsu et al., 2015a). We did not observe significant differences between GP.Mur-positive and GP.Mur-negative groups in exercise-induced changes of heart rates, %O\(_2\) saturation, and lactate production.

We also noticed that the changes of blood CO\(_2\) and bicarbonate due to this mild exercise challenge were smaller in GP.Mur-positive subjects (Hsu et al., 2015a). One's blood gas levels are tuned by his or her breathing rhythm, which is regulated by the respiratory centers, or the central controller neurons located in medulla and pons of the brainstem (West, 2005). Since the efficiencies of CO\(_2\) respiration are differentiable by GP.Mur phenotype, the neurological responses from the brainstem respiratory centers are expected to be differentiable (Hsu et al., 2015a).

**Figure 2** summarizes the impacts of differential band 3 expression on blood CO\(_2\) transport. At one extreme with little or no band 3 expression, the lack of erythroid AE1 appears lethal for most mammalian species, except a breed of Japanese cattle who could grow to adulthood with severe systemic defects (Inaba et al., 1996). The red cell membrane from band 3-deficient animals is very unstable and fragile, resulting in spherocytosis and severe anemia. Band 3 knockout substantially dissipates most of the DIDS-sensitive anion transport, resulting in acidosis and reduced anion exchange and CO\(_2\) transport (Inaba et al., 1996; Peters et al., 1996; Southgate et al., 1996). The other end of the spectrum is represented by GP.Mur, an example of higher band 3 expression. The higher densities of band 3 in GP.Mur erythrocytes strengthen membrane stability, and enlarge the capacity of anion exchange. Higher band 3 expression manifests systemically in more efficient clearance of CO\(_2\) (Hsu et al., 2009, 2015a). Perhaps not coincidentally, several Taiwanese tribes with highest rates of GP.Mur prevalence in the world (20–90% GP.Mur-positive) have long been recognized for superior physical performance, endurance, and even athleticism, compared to other ethnic groups in Taiwan (1–2% GP.Mur-positive) (Shikakogi, 1912/1985; Wu and Hu, 1999; Aoki, 2002; Kuo, 2007; Lai, 2013).
OSMOTICALLY SENSITIVE INTERACTION BETWEEN AQP1 AND BAND 3

Mammalian CO₂ transport relies on constant hydration of CO₂ and dehydration of HCO₃⁻ inside erythrocytes. Approximately 0.1% of unbound, intraerythrocytic water is estimated to be used for CO₂/HCO₃⁻ conversion, after excluding maximally 85% of intraerythrocytic water that is structured or bound H₂O (0.34–1.44 g structured H₂O/g dry mass in intact erythrocytes) (Cameron et al., 1988). Intraerythrocytic H₂O can be considered as an unlimited substrate. But unexpectedly, our early study found significantly more AQP1-band 3 protein complexes in GP.Mur-positive than in GP.Mur-negative RBCs, despite that the protein levels of AQP1 are the same in both GP.Mur-positive and GP.Mur-negative erythrocytes (Hsu et al., 2009). Does this suggest that the functionality of AQP1 is associated with the functionality of band 3? Or is this AQP1-band 3 interaction unique to GP.Mur-expressed cells?

We later employed a sensitive, biophysical approach—fluorescence resonance energy transfer by fluorescence lifetime imaging microscopy (FLIM-FRET), to verify AQP1-band 3 interaction that was initially identified by proteomics (Hsu et al., 2009). FLIM measures the fluorescence lifetimes of single fluorophore molecules. FRET, a phenomenon that only occurs within the range of dipole–dipole interaction (10 nm), can be measured by FLIM with much less bias than traditional intensity-based measurements. By this approach, AQP1 and band 3 are found at a distance of 8 nm from each other on the erythrocyte membrane. Though their interaction is more obvious in GP.Mur RBCs, AQP1-band 3 interaction also exists in RBCs lacking GP.Mur. Importantly, this AQP1-band 3 interaction could be dissipated by hypotonic conditioning (e.g., by diluting the physiological buffer HBSS with water to ∼250 mOsm/Kg) (Hsu et al., 2017). This osmotically sensitive interaction between AQP1 and band 3 conceivably allows erythrocytes to sense and respond to hemodynamic shear stress, as well as changes of erythrocyte volume and shape during circulation. Thus, AQP1-band 3 interaction on the red cell membrane is dynamic, not static. The protein–protein interaction between GPA and band 3 is also dynamic and changeable by essential cellular processes, such as phosphorylation of band 3 (Giger et al., 2016).

REVISIT THE MODEL—“CO₂-TRANSPORT METABOLON”

Band 3, CAII, and AQP1 are all functionally associated with one another for intraerythrocytic CO₂/HCO₃⁻ conversion. After the discovery of the protein–protein interaction between CAII and band 3 (Vince and Reithmeier, 2000; Sterling et al., 2001a,b), the band 3-CAII protein complex has been proposed to be the core of a CO₂-transport metabolon because of their structural-functional correlation (Reithmeier, 2001; Bruce et al., 2003; Sowah and Casey, 2011). Recently, AQP1 has also been demonstrated to be spatially associated with CAII, and to transport H₂O for CAII-mediated catalysis (Vilas et al., 2015). Nonetheless, the existence of a CO₂-transport metabolon was challenged in two studies, where the protein–protein interaction between band 3 and CAII could not be identified either by a tsA201 (SV40-transformed HEK) heterologous expression system, or by the direct binding assay with individual purified proteins (Piermarini et al., 2007; Al-Samir et al., 2013).

Perhaps the interactions between band 3 and other red cell proteins are much more complicated than we initially envisioned. Band 3 protein complexes are currently categorized into three types, with differences in their individual components and in their distinct cellular localization relative to the submembranous spectrin network. About half of band 3 molecules are anchored submembranously onto specific sites of the spectrin network in dimeric forms (junctional complexes) and tetrameric forms (ankyrin complexes); the other half of band 3 molecules (~640,000 copies/RBC) are mobile dimers located within the corrals set by the submembranous spectrin mesh (Steck, 1978; Burton and Bruce, 2011). AQP1 is confined to the corrals of the underlying cytoskeletal network (Cho et al., 1999). Conceivably, membrane-bound, mobile AQP1 and cytosolic CAII may preferentially interact with one or more types of the band 3 complexes, resulting in structurally and functionally differentiable complexes on the erythrocyte membrane. Very likely the population of cytoskeleton-independent band 3 preferentially interacts with AQP1, as the protein–protein interaction between AQP1 and band 3 was measured by FLIM-FRET using inside-out vesicles (IOVs) from erythrocyte membrane that were depleted of much spectrin cytoskeleton and non-integral membrane-bound proteins (Hsu et al., 2017). Additionally, as AQP1-band 3 interaction is adaptable to osmotic changes (Hsu et al., 2017), it is possible that the interaction between CAII and band 3 could also be transitory and sensitive to osmotic or hemodynamic shear stresses (Lazaro et al., 2014).

INFERRED FROM OSMOTICALLY SENSITIVE AQP1-BAND 3 INTERACTION: “CO₂-H₂O ON DEMAND” IN THE CO₂-TRANSPORT METABOLON

The idea of a CO₂-transport metabolon, first proposed by Reithmeier, is based on the experimental observation that the major proteins supporting this intraerythrocytic reaction (CO₂ + H₂O ⇌ HCO₃⁻ + H⁺) are spatially adjacent to one another to maximize the efficiency of blood CO₂ transport (Figure 3) (Reithmeier, 2001). This forward reaction takes place when red cells circulate to capillaries surrounded by tissues, where O₂ fluxes out of RBCs and CO₂ fluxes in. Deoxygenated hemoglobin (deoxy Hb) preferentially binds to the N-terminal, cytoplasmic domain of band 3 (Walder et al., 1984; Castagnola et al., 2010; Chu et al., 2016). The forward reaction is driven by the removal of the two products: (1) band 3-mediated export of HCO₃⁻; (2)
absorption of proton by negatively charged deoxy Hb which is transiently bound to band 3.

It is estimated that ~60% of CO₂ flux in or out of RBCs is via AQP1 gas channel. The rest of CO₂ flux is likely through another gas channel, RhAG, and/or direct diffusion across the lipid bilayer (Blank and Ehmke, 2003; Endeward et al., 2006, 2008; Wang et al., 2007; Boron, 2010). The transient association between AQP1 and band 3/CaII/deoxy Hb conceivably enables the formation of a spatially connected passage for each step of CO₂/HCO₃⁻ exchange, when erythrocytes circulate to systemic capillaries: (1) the entry of the substrates CO₂ and H₂O into RBCs through AQP1, (2) intraerythrocytic hydration of CO₂ by CaII, (3) the exit of one of the two reaction products, bicarbonate, from erythrocytes via band 3, and (4) the absorption of the other reaction product, proton, by nearby deoxy Hb⁻ (Chu et al., 2016) (Figure 3). This transient arrangement of having H₂O/CO₂ channel AQP1, adjacent to band 3, CaII and proton-absorbing deoxy Hb⁻, conceivably allows an almost uninterrupted “channeling” of reactant influx and product outflow (Moraes and Reithmeier, 2012). The transient structural coupling also allows CO₂/HCO₃⁻ exchange to be primarily carried out near the surface and the submembranous zone of erythrocytes whenever possible, which may further support the efficiency of CO₂ transport.

From the enzymatic perspective, the active site of CaII is conically shaped and lined with ordered water molecules (Liang and Lipscomb, 1990). This network of ordered water molecules supports rapid proton transfer (Mikulski et al., 2013), which is rate-limiting for CaII-mediated catalysis. By supporting the formation of “low barrier H⁺-bond” (LBHB), the network of ordered water allows CO₂ loosely bound to CaII (Krebs et al., 1993). This weak substrate (CO₂) binding allows a nearly complete occupancy of CO₂ at the active site, contributing to the ultra-high efficiency of CaII. Indeed, the concentration of CO₂ at the active site of CaII is very high (0.45 M) (Krebs et al., 1993; Domsic et al., 2008). This implies that the CaII-extended machinery (or the channel built by transient linkages of AQP1, band 3, CaII, and deoxy Hb) could function with a mode of “CO₂-H₂O on demand,” since the two reaction substrates—CO₂ and H₂O, are always near complete saturation in the active site of CaII. Conceivably, this design of “CO₂-H₂O on demand” could increase the sensitivity of circulating erythrocytes to blood CO₂ gradients, as even a very small change in the concentration of the substrate could trigger CaII-mediated catalysis.

**THE POTENTIAL ROLE OF AQP1 IN BLOOD CO₂ RESPIRATION**

AQP1 as a H₂O/CO₂ channel is functionally replaceable by other channels/transporters (Iserovich et al., 2002; Yang and Verkman, 2002; Zeuthen et al., 2016). The first clue is that AQP1 null people (of Colton blood types) appear healthy (Preston et al., 1994). This strongly hints that its H₂O/CO₂-transporting function might not be exclusively carried out physiologically by AQP1 alone. The only known symptom in AQP1 null people shows upon water deprivation; AQP1 deficiency reduces their capability to concentrate urine or reabsorb free water at the medullary collecting duct of the kidney (King et al., 2001). Similarly, AQP1-deficient mice show normal survival and reduced ability in urine concentration (Ma et al., 1998).

The second clue came from comparing the single knockouts of AQP1 or urea transporter UT-B to the double knockout of AQP1 and UT-B. The RBCs from AQP1 null mice exhibit significantly reduced osmotic H₂O permeability. The RBCs from UT-B null mice are similarly water-permeable as the RBCs from wild-type mice. But water permeability in the RBCs of AQP1 and UT-B double-knockout mice is further reduced. Double knockout of AQP1 and UT-B show reduced survival and retarded growth, in addition to reduced urinary concentrating ability (Yang and Verkman, 2002). This suggests that urea transporter does not primarily function to permeate water, but it can transport water when needed. Another RBC membrane transporter—glucose transporter GLUT1, is also capable of permeating water (Iserovich et al., 2002; Zeuthen et al., 2016). Therefore, AQP1 is the main water channel in human (especially erythrocytes), but its function in water permeation can be alternatively supported by urea transporter and glucose transporter (Iserovich et al., 2002; Yang and Verkman, 2002; Zeuthen et al., 2016).

The CO₂-transporting function of AQP1 is replaceable by RhAG gas channel (Ripoche et al., 2006; Endeward et al., 2008; Musa-Aziz et al., 2009). The functional overlaps between
AQP1 and RhAG may explain why defective CO$_2$ transport is not observed in AQP1 knockout mice (Yang et al., 2000). Interestingly, recent new findings from AQP1 knockout mice suggest that AQP1 expression enhances one’s tolerance or endurance for extreme physical activities (Xu et al., 2010; Al-Samir et al., 2016a,b). Their results somehow resonate with our findings from studying the GP.Mur phenotype, which suggest potential involvement of AQP1 in blood CO$_2$ respiration and physical tolerance. Unlike the clear-cut monogenic effect of kidney AQP1 in urine concentration, the role of AQP1 in red cell functions remains to be explored.

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