BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

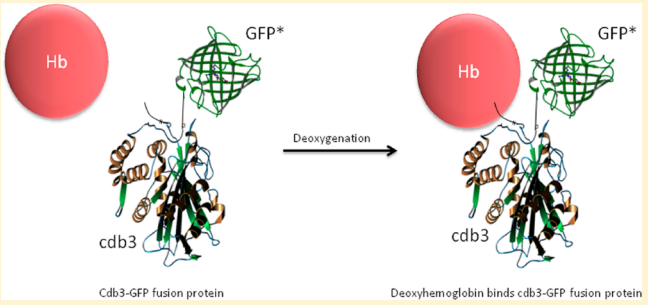
Interaction of Deoxyhemoglobin with the Cytoplasmic Domain of Murine Erythrocyte Band 3

Martiana F. Sega,[†,‡] Haiyan Chu,[†] John Christian,[‡] and Philip S. Low*[,†]

[†]Department of Chemistry and [‡]Comparative Pathobiology, Purdue University, West Lafayette, Indiana 47907, United States

Supporting Information

ABSTRACT: The partial pressure of oxygen constitutes an important factor in the regulation of human erythrocyte physiology, including control of cell volume, membrane structure, and glucose metabolism. Because band 3 is thought to be involved in all three processes and because binding of hemoglobin (Hb) to the cytoplasmic domain of band 3 (cdb3) is strongly oxygen-dependent, the possibility that the reversible association of deoxyhemoglobin (deoxyHb) with cdb3 might constitute an O_2-dependent sensor that mediates O_2-regulated changes in erythrocyte properties arises. While several lines of evidence support this hypothesis, a major opposing argument lies in the fact that the deoxyHb binding sequence on human cdb3 is not conserved. Moreover, no effect of O_2 pressure on Hb−band 3 interactions has ever been demonstrated in another species. To explore whether band 3−Hb interactions might be widely involved in O_2-dependent regulation of erythrocyte physiology, we undertook characterization of the effect of O_2 on band 3−Hb interactions in the mouse. We report here that murine band 3 binds deoxyHb with significantly greater affinity than oxyHb, despite the lack of significant homology within the deoxyHb binding sequence. We further map the deoxyHb binding site on murine band 3 and show that deletion of the site eliminates deoxyHb binding. Finally, we identify mutations in murine cdb3 that either enhance or eliminate its affinity for murine deoxyHb. These data demonstrate that despite a lack of homology in the sequences of both murine band 3 and murine Hb, a strong oxygen-dependent association of the two proteins has been conserved.



Considerable evidence demonstrates that multiple erythrocyte properties are regulated by the partial pressure of oxygen to which the red cells are exposed. Among the functions thought to be controlled by O_2 levels are glucose metabolism, cell volume and hydration, and membrane structure.[1−5] Erythrocyte glucose consumption occurs primarily via glycolysis in deoxygenated cells, but upon exposure to O_2, a considerable fraction of the cell's glucose is channeled into the pentose phosphate pathway.[6−8] The importance of this regulatory switch has been suggested to lie in the heightened need for reductants during periods of elevated levels of exposure to O_2 to protect the cell against oxidative stress.[2,9] Thus, by activating the pentose phosphate pathway upon erythrocyte oxygenation, the cell is assured of sufficient NADPH for both glutathione reduction and maintenance of Hb in its reduced state.

What is the evidence of band 3−deoxyHb interactions in this regulation? Data from other laboratories and our own show that the glycolytic enzymes bind avidly to the NH_2 terminus of band 3.[10−14] These data also demonstrate that deoxyHb (but not oxyHb) competes avidly for this enzyme binding site on human band 3,[3] and that upon red cell deoxygenation, the vast excess of deoxygenated Hb competitively displaces glycolytic enzymes from the membrane.[15,16] Because the catalytic properties of the glycolytic enzymes are significantly altered upon association with band 3,[13,17−19] reversible displacement of these enzymes by deoxyHb can explain the O_2-dependent switch in red cell metabolism. However, as noted above, the lack of homology between the deoxyHb binding site on human and other mammalian band 3 orthologs raises questions about the validity of the proposed regulatory mechanism.

Evidence of the role of band 3−deoxyHb interactions in the regulation of red cell volume or hydration by oxygen pressure is also mounting. To facilitate volume modulation during transit through regions of hypotonic or hypertonic stress, erythrocytes are equipped with an array of cotransporters that can reverse either cell swelling or cell shrinkage upon activation.[20−22] Importantly, the K^+/Cl^- cotransporter (KCC) in human erythrocytes undergoes an ∼20-fold increase in activity during erythrocyte oxygenation.[23] Moreover, this O_2-dependent regulation occurs only in whole cells and Hb-containing ghosts, not in white ghosts or whole cells treated with CO to block O_2 binding.[24,25] Together with data showing a sigmoidal dependence of K^+/Cl^- cotransport on O_2 pressure (i.e., similar to the sigmoidal dependence of Hb saturation on O_2 pressure), the results suggest that Hb must participate in the O_2-dependent switch in KCC activity.[22,26] Because band 3 constitutes the only established binding site for deoxyHb on the membrane,[3] the participation of band 3 in the O_2-triggered KCC regulation has frequently been proposed.[21,22,27] Similarly, an O_2-dependent

Received: October 25, 2011
Revised: March 23, 2012
Published: March 27, 2012

   dx.doi.org/10.1021/bi201623v | *Biochemistry* 2012, 51, 3264−3272

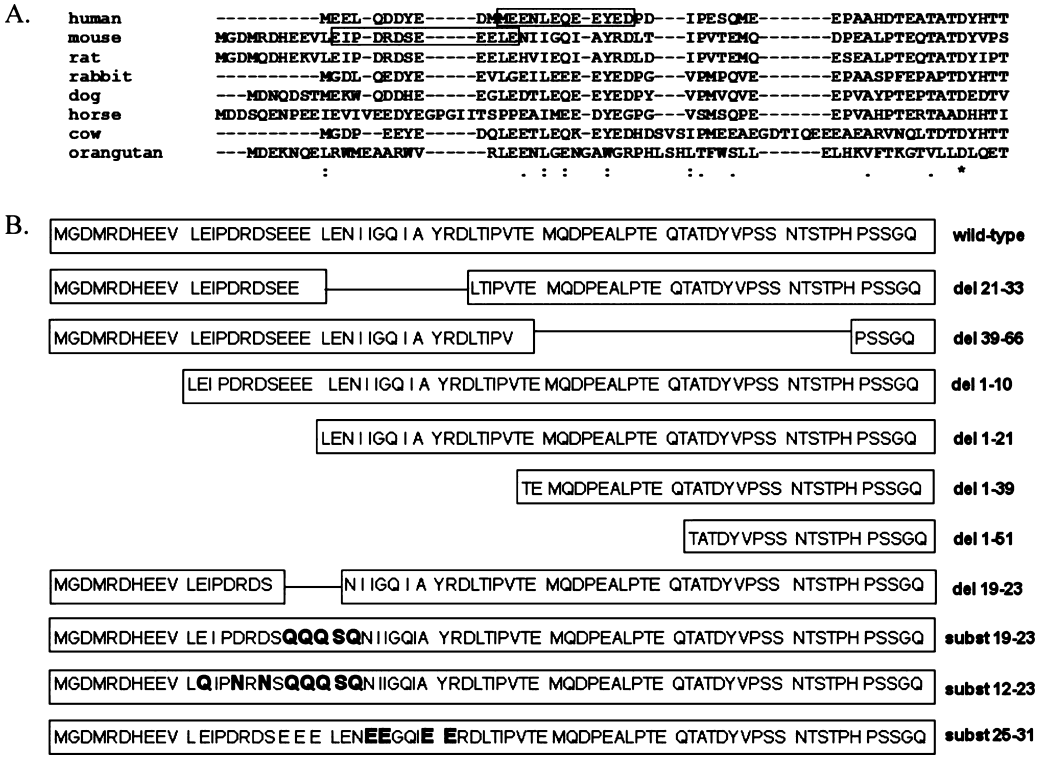


Figure 1. (A) Alignment of the NH_2 terminus of band 3 from different mammalian erythrocytes. The experimentally established deoxyhemoglobin binding sites for human and murine band 3 are framed in boxes. The sequence alignment was performed using the CLUSTAL W (http://workbench.sdsc.edu/). Asterisks indicate complete sequence conservation, colons conservation of strong groups, and periods conservation of weak groups. (B) Description of the mutant cytoplasmic domains of band 3 used in this study. For simplicity, only the first 70 amino acids of 398-residue mouse cdb3 are shown. Deletions are denoted del and substitutions subst. The substituted residues are in bold.

change in sickle cell cation transport (termed P_{sickle}) has been observed, as have O_2-triggered changes in the activities of the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/H^+ antiporter.[28−30] However, once again, the absence of homology in the critical band 3−deoxyHb binding site casts doubt on the universality of the participation of band 3 in the proposed mechanism.

Finally, evidence that human erythrocytes might also modulate their membrane structural properties in response to changes in O_2 tension is emerging. During their ∼120 day life span, red blood cells continuously squeeze through capillaries or sinusoids that are less than half their cell diameters. Their ability to recover their biconcave shape following exit from these depends at least in part on interactions between the plasma membrane and its underlying spectrin-based membrane skeleton.[31] Importantly, band 3 constitutes a major anchor for the spectrin skeleton on the membrane, and ankyrin performs the major bridging function that connects band 3 to spectrin.[32] Because the band 3−ankyrin interaction has recently been shown to be O_2-sensitive (M. Stefanovic and P. Low, unpublished data), the possibility that displacement of ankyrin from band 3 by deoxyHb might serve as an O_2 switch that can confer O_2 sensitivity on red cell rheology arises.

Figure 1A shows the sequence on human band 3 that was previously demonstrated to bind human deoxyHb (boxed residues). Not only does deletion of this sequence abrogate binding of deoxyHb to human band 3, but mutations on either side of this sequence have also been found to elevate the affinity of deoxyHb for human band 3 so dramatically that deoxyHb remains bound even at supraphysiological O_2 pressures.[3] Thus, the deoxyHb binding site appears to allow the reversible O_2-dependent association of band 3 and deoxyHb precisely over the physiological range of O_2 pressures. To explore whether this O_2-dependent binding function might have been maintained in other mammalian species despite obvious differences in primary structure, we have examined whether murine band 3 might display preferential affinity for murine oxyHb or deoxyHb. We have also mutated many of the residues within the NH_2 terminus of murine band 3 to determine whether murine Hb affinity might be affected. We report here that a nonhomologous sequence near the NH_2 terminus of murine band 3 selectively binds murine deoxyHb and that mutations within and adjacent to this sequence generate band 3 mutants with either high or very low affinity for mouse deoxyHb.

EXPERIMENTAL PROCEDURES

Materials. Protease inhibitors were purchased from Research Products International Corp. All protein concentration steps were performed by ultrafiltration using Vivaspin tubes from GE Healthcare Life Sciences. Dialysis was performed with dialysis bags from Spectrum Laboratories Inc. The protein concentration was measured with a MicroBCA protein assay from Thermo Scientific. All others materials and reagents were purchased from Sigma-Aldrich.

GFP Fusion Constructs. One assay for measuring the affinity of murine deoxyHb for murine band 3 involved preparation of a fusion protein between the cytoplasmic domain of band 3 (cdb3) and GFP, and measuring the quenching of this fusion construct upon its association with Hb. The GFP fusion vector employed was a generous gift from D. Thompson (Purdue University). GFP containing a histidine tag (His_8) attached to its own COOH terminus was fused to the

dx.doi.org/10.1021/bi201623v | Biochemistry 2012, 51, 3264−3272

COOH terminus of wild-type and mutant cdb3 proteins. Murine cdb3 DNA (encoding amino acids 1−398) was amplified via polymerase chain reaction (PCR) using a forward primer containing an NdeI cleavage site followed by a start codon (5′-cat atg ggg gac atg cgg gac cac-3′) and kidney cdb3 (lacking the first 79 amino acids) using the forward primer 5′-gcg cat atg gac cag agg aac cag-3′; the reverse primer was 5′-ctc gag aaa gat ccg gcc tgt gcg-3′. Because the GFP gene has an additional NdeI cleavage site in its sequence, we mutated this site with conservative substitutions, yielding a final construct containing only the single NdeI site in the multiple-cloning site. The wild-type and kidney murine cdb3 sequences were fused to the NH_2 terminus of GFP proteins between the NdeI and XhoI enzymatic sites. The amplified DNA product was first ligated into a pGEM-T easy vector (Promega) and then excised with NdeI and XhoI from the pGEM-T vector. After the product had been ligated into the GFP fusion vector, the final construct was sequenced to ensure the absence of unwanted mutations.

Wild-type murine cdb3 cDNA lacking GFP and His tags was prepared by PCR amplification using the same parent cDNA described above and inserted into the pGEM-T vector. After digestion with NdeI and HindIII, the sequence was inserted into a pT-7.7 vector for protein expression.

Site-Directed Mutagenesis. All mutated proteins were prepared using a QuickChange mutagenesis kit (Stratagene) following the manufacturer's instructions. The sense primers used in this study were as follows: for deletion of residues 1−51 (del 1−51), 5′-gga gat ata cat atg aca gcc aca gac tac gtc-3′; for deletion of residues 1−38 (del 1−38), 5′-ggg tcc tgc atc tcg gtc ata tgt ata tct cc-3′; for deletion of residues 1−21 (del 1−21), 5′-gga gat ata cat atg ctg gag aac ata ata gga cag ata gc-3′; for deletion of residues 1−10, 5′-gat ata cat atg ctg gag atc ccag-3′; for deletion of residues 19−23, 5′-gat cga gac agc aac ata ata ggac-3′; for deletion of residues 21−33, 5′-cga gac agc gaa gaa cta acc atcc-3′; for deletion of residues 39−66, 5′-cta acc atc cct gt g agc tcc ggt ca-3′; for substitution of residues 19−23, 5′-cca gat cga gac agc caa caa caa tcg cag aac ata ata gga cag-3′; for substitution of residues 12−23, 5′-gga agt gct gca gat ccc aaa tcg aaa cag cca aca ac-3′; for substitution of residues 25−31, 5′-gaa ctg gag aac gaa gaa gga cag ata gaa gat aga gac cta acc-3′. The 12−23 substitution mutant was prepared using the 19−23 substitution mutant DNA as the template. All other constructs were made using the wild-type cdb3−GFP fusion DNA as a template. Sequences of the final products were all confirmed by DNA sequencing.

Expression and Purification of Proteins. GFP fusion proteins were expressed using BL21(DE3)pLysS bacterial strains (Invitrogen) in 2XYT medium at 28 °C for 2−3 h. The collected pellet was stored overnight at −80 °C and then thawed in lysis buffer [20 mM NaH_2PO_4, 500 mM NaCl, 15 mM imidazole, and 1 μM AEBSF (pH 7.5)] at room temperature and lysed using a French press (SLM-Aminco). The bacterial lysate was clarified by centrifugation at 17000*g* on a Sorval SS-35 rotor for 30 min, and the supernatant was filtered and loaded onto a nickel affinity column equilibrated in lysis buffer (GE Healthcare Life Sciences). The column was first washed with lysis buffer containing 40 mM imidazole and then eluted with the same buffer containing 250 mM imidazole.

Proteins lacking GFP fusion constructs and affinity tags were expressed in BL21(DE3)pLysS bacteria (Invitrogen) in 2XYT medium at 29 °C for 3−4 h. Bacterial pellets were lysed in 20 mM Tris, 1 mM EDTA, 0.2% Triton X-100, and 0.2% β-mercaptoethanol (pH 7), and wild-type and mutated cdb3 proteins were purified by anion exchange chromatography and hydrophobic column chromatography, as described previously.[3]

Hemoglobin Purification and Handling. Hemoglobin for all cdb3−GFP binding studies was obtained from erythrocytes of C57BL/6J mice, as described previously.[33] Hemoglobin from C57BL/6 mice is highly homogeneous and does not polymerize when stored as other murine hemoglobins do.[34] When larger quantities of blood were required for the HEMOX analyses, hemoglobins from C57BL/6J and BALB/cJ mice were mixed in a 1.2:1 ratio prior to analysis.

For Hb isolation, whole blood was pelleted and the buffy-coat and plasma were removed by repeated washing in PBS [137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_2PO_4, and 1.5 mM KH_2PO_4 (pH 7.4)]. The packed red blood cells were stored at 4 °C for 1 week to begin depletion of 2,3-diphosphoglycerate (2,3-DPG), after which the cells were lysed in 5 volumes of cold water and spun at 20000*g*. To remove any residual 2,3-DPG, the supernatant was first dialyzed for 12 h in 1 M NaCl, then in 0.1 M NaCl, and finally in 10 mM BisTris acetate (pH 6.5). The hemoglobin concentration was determined by measuring the absorbance at 540 nm using an extinction coefficient of 14.17.[35] Deoxygenation was performed by flushing the Hb solution with humidified argon gas until the change in the absorption spectrum confirmed deoxygenation of the sample.

Fluorescence Resonance Energy Transfer Analysis of the Affinity of DeoxyHb for Band 3. All fluorescence measurements were taken on an AMINCO-Bowman luminescence spectrometer. The excitation wavelength for analysis of cdb3−GFP fusion protein solutions was set at 386 nm, and the emission spectrum was scanned from 470 to 550 nm. Both murine cdb3 mutants (0.5 μM) and hemoglobin (1 μM) were dialyzed against 10 mM BisTris acetate buffer (pH 6.5) at room temperature before analysis of binding. Samples were incubated for 5 min at room temperature prior to measurement of the GFP quenching by any bound oxyHb. The emission spectrum of a fully oxygenated solution of the cdb3−GFP fusion protein and Hb was recorded first, after which the solution was deoxygenated and the emission spectrum recorded again. In most cases, the reversibility of the process was confirmed by reoxygenating the sample. In addition, a control sample containing pure GFP (not fused to cdb3) was analyzed to allow subtraction of the inner filter effect using the following formula:

$$\%\ \text{Hb quenching} = \left(1 - \frac{F_{\text{protein+dHb}}}{F_{\text{gfp+dHb}}}\right) \times 100$$

where F is the fluorescence of the GFP-fused protein or pure GFP in the presence of deoxyHb.

Equilibrium Binding Analysis of the Affinity of DeoxyHb for Band 3. To measure the equilibrium dissociation constant for the interaction of deoxyHb with different mutated forms of cdb3-GFP, increasing concentrations of deoxyHb were incubated for 45 min at room temperature with cdb3-GFP (or one of its mutants) in BisTris acetate buffer, after which the intensity of GFP fluorescence was measured as described above. Fluorescence quenching was then calculated using the equation given above on three separate replicates at each deoxyHb concentration (on two different days), and the mean and standard deviations were determined at each deoxyHb concentration. GraphPad Prism version 4.00 for Windows was then used to fit the plot of

fluorescence quenching as a function of deoxyHb concentration to a simple single-site noncooperative binding curve and calculate the equilibrium dissociation constant.

Analysis of The Affinity of DeoxyHb for Band 3 by Measurement of Oxygen Dissociation Curves. Preferential binding of deoxyHb over oxyHb to cdb3 can be assessed by measuring the shift in the oxygen dissociation curve of Hb in the presence of cdb3. For this purpose, hemoglobin and wild-type or mutant cdb3 were dialyzed against 10 mM BisTris acetate buffer (pH 6.5), and 20 nmol of Hb was incubated with 10 nmol of cdb3 for 1 min at 37 °C; 20 μL of a 20% BSA solution and 10 μL of antifoam solutions (TCS, Southampton, PA) were then added to the cdb3/Hb solution, and oxygen–Hb dissociation curves were generated on a HEMOX analyzer (TCS) according to the manufacturer's instructions. The effect of cdb3 on the affinity of Hb for oxygen is reported as a shift in the P_{50} value for Hb.

Data Analysis. All average results are presented as means ± the standard error. The graphs were made using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Murine DeoxyHb Exhibits a Higher Affinity Than OxyHb for Murine cdb3 . As discussed in the introductory section, modulation of cdb3–hemoglobin interactions by O_2 has been proposed to account for oxygen control of several erythrocyte properties, including cell hydration, glucose metabolism, and membrane structure.[1−3] However, because the sequence on human band 3 responsible for Hb binding (i.e., the NH_2 terminus of band 3) is not highly conserved (see Figure 1A), the question of whether the deoxyHb–band 3 interaction could constitute the O_2 sensor that would mediate the effects of O_2 on erythrocyte physiology across species naturally arose. To directly test this hypothesis and on the basis of our crystallographic data[36] that showed both N- and C-termini very close to each other, we chose to generate a fusion protein with the COOH terminus of murine cdb3 and GFP and examine the binding of oxyHb and deoxyHb to this fusion construct by quantitating the quenching of GFP fluorescence. As seen in Figure 2A, murine Hb displays O_2-dependent binding to murine cdb3, with deoxyHb exhibiting a higher affinity for cdb3 than oxyHb. As a negative control, the kidney spliceoform of murine cdb3 (kcdb3) that lacks the first 79 amino acids, including the Hb binding site, displays little affinity for Hb under either oxygenated or deoxygenated conditions. These results indicate that O_2 tension constitutes a modulator of cdb3–Hb interactions in the mouse despite the lack of significant homology in the region previously shown to bind Hb in the human.

The Deoxyhemoglobin Binding Site on Mouse cdb3 Is Primarily Located within the First 21 Residues of the Anion Transporter. The sequence alignment showed residues 21–33 of murine cdb3 to be most similar to amino acids 12–23 of human cdb3, i.e., the deoxyHb binding site on human cdb3 (see ref 3, as indicated by the box in Figure 1A). To test whether residues 21–33 of murine cdb3 indeed comprise the deoxyHb binding site on murine cdb3, these residues were deleted and deoxyHb binding affinity was analyzed by measuring the O_2 dissociation curve. As seen in Figure 2B, only a small reduction in the level of deoxyHb binding ensued, suggesting that residues 21–33 of murine cdb3 do not contain the major residues involved in Hb binding.

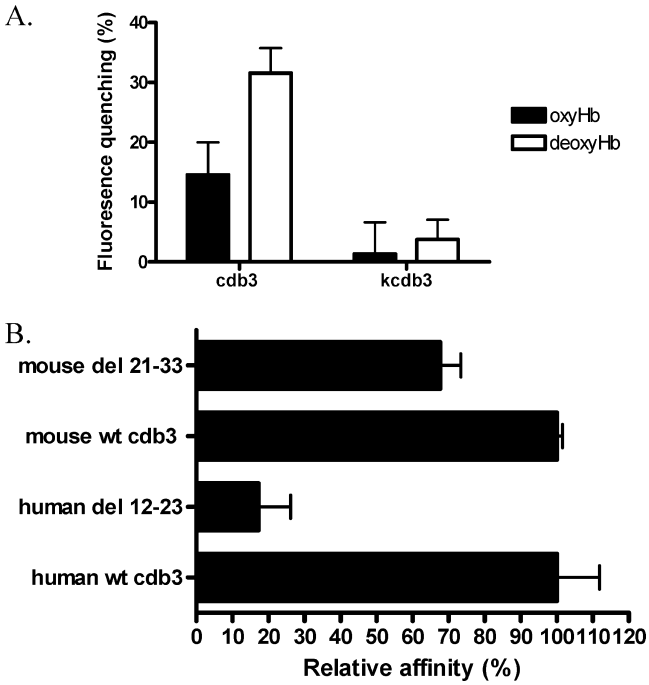


Figure 2. (A) Relative affinity of murine oxy- and deoxyHb (1 μM) for murine cdb3 (0.5 μM) determined by fluorescence quenching. Because the kidney spliceoform of cdb3 (kcdb3) lacks the entire NH_2 terminus (residues 1–79), it was used as a nonbinding (negative) control. (B) Effect of deletion from murine cdb3 of the sequence homologous to the deoxyHb binding site on human cdb3 on the affinity of the mutated murine cdb3 for murine deoxyHb. On the basis of the sequence alignment in panel A, the sequence in murine cdb3 (residues 21–33) found to be most homologous to the deoxyHb binding site on human cdb3 (residues 12–23) was deleted and its relative binding affinity for murine deoxyHb was examined by measuring the shift in the oxygen dissociation curve in the presence of cdb3. For comparison, the relative affinity of human deoxyHb for human cdb3 is also shown.

To begin to identify those residues that might be critical for deoxyHb binding in the mouse, we prepared a series of deletion and substitution mutants with mutations near the NH_2 terminus of murine cdb3 (Figure 1B) and re-examined each mutant's affinity for deoxyHb. As seen in Figure 3A (see also the raw data in Figure S3 of the Supporting Information), deletion of either amino acids 21–33 or 39–66 exerts little effect on GFP fluorescence, suggesting that residues 21–66 do not participate significantly in deoxyHb binding. In contrast, a dramatic decrease in the level of fluorescence quenching is seen upon deletion of any NH_2-terminal fragment containing residues 1–21. These data suggest that the murine deoxyHb binding site resides within the first 21 amino acids of the cdb3 polypeptide (Figure 3A).

The Deoxyhemoglobin Binding Site on Murine cdb3 Resides between Amino Acids 12 and 23. On the basis of the fact that cdb3–deoxyHb binding is predominantly electrostatic in nature,[14,37] and the negatively charged amino acids $E^{19}EYED^{23}$ were shown to play a critical role in human deoxyHb binding to human cdb3,[3] we next decided to explore whether the murine sequence $E^{19}EELE^{23}$ might be similarly involved in murine deoxyHb binding. For this purpose, we either deleted the sequence (del 19–23) or replaced each acidic residue with its uncharged amide counterpart [subst 19–23 (Figure 1B)]. Moreover, because the first 10 amino acids in

dx.doi.org/10.1021/bi201623v | *Biochemistry* 2012, 51, 3264–3272

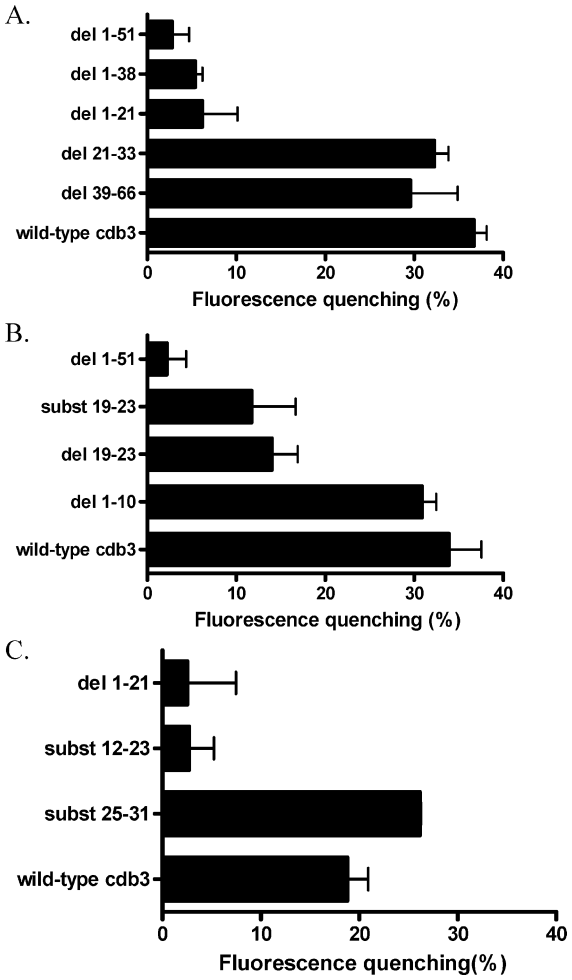


Figure 3. (A) Comparison of the relative affinities of different murine cdb3 mutants for murine deoxyHb. For analysis of deoxyHb binding affinity, the COOH terminus of each cdb3 was fused to GFPuv and the binding of deoxyHb was assayed by quantitating the quenching of GFPuv fluorescence upon deoxyHb binding. In all cases, the inner filter effect due to Hb absorption of GFPuv fluorescence was subtracted (see Experimental Procedures). As seen, mutant proteins that lack the first 21 amino acids do not bind deoxyHb (i.e., GFP fluorescence is high for del 1−51, del 1−38, and del 1−21), but when these first 21 residues are present (wild type, del 21−33, and del 39−66), binding affinity is high. A del 1−51 mutant was used as a negative control, because it exhibits no affinity for deoxyHb. See Figure 1 for a description of the mutations. (B) Localization of the deoxyHb binding site on murine cdb3 to residues 12−23. (C) Examination of substitution mutations within the binding region comprising residues 12−23 of cdb3.

murine cdb3 also include four acidic residues (see Figure 1B), we also investigated whether deletion of these residues might similarly weaken deoxyHb binding (del 1−10). The results of these studies are presented in Figure 3B. As shown in the bar graphs, removal or replacement of acidic amino acids between amino acids 19 and 23 (see del 19−23, and subst 19−23) yields a cdb3 with significantly reduced affinity for deoxyHb, suggesting that negatively charged residues within the 19−23 peptide contribute prominently to deoxyHb binding. Surprisingly, however, deletion of residues 1−10 with their accompanying four acidic residues exerts little effect on deoxyHb affinity (Figure 3B, del 1−10). Taken together, these findings suggest that the binding site of murine deoxyHb on murine cdb3 does not reside within the first 10-mer fragment but rather is centered in the directly adjacent region comprising residues 19−23 of mouse cdb3.

Because removal or replacement of the four acidic amino acids between residues 19 and 23 resulted in an only 60% reduction in the level of deoxyHb binding (Figure 3B), and the deoxyHb binding peptide in human band 3 comprised 7−12 amino acids, we elected to determine whether elimination of additional negative charges near the NH_2 terminus of cdb3 might further reduce deoxyHb affinity. For this purpose, all seven acidic amino acids between residues 12 and 23 were converted to their corresponding amides (Figure 1B, subst 12−23), and deoxyHb binding was again examined. As seen in Figure 3C, removal of the aforementioned additional negative charges further lowered the affinity of cdb3 for deoxyHb to 15% of the normal value. These data suggest that the major docking site of murine deoxyHb on murine cdb3 resides primarily between amino acids 12 and 23.

Identification of a Mutant Murine cdb3 with a Higher Affinity for Murine DeoxyHb Than Wild-Type Murine cdb3. The finding that certain mutations in human cdb3 led to a polypeptide with little affinity for deoxyHb while other mutations generated a band 3 with such high affinity that even supraphysiological pressures of O_2 could not promote its dissociation from deoxyHb[3] suggests that the O_2 dependence of Hb−cdb3 binding occurs precisely over the physiological range of oxygen pressures. To determine whether murine cdb3 might have a similar sensitivity to physiological O_2, we looked for mutations in cdb3 that might elevate its deoxyHb affinity to a level that would prevent deoxyHb dissociation even at supraphysiological O_2 pressures. Identification of such a mutation would support the contention that O_2 regulation of Hb binding in the mouse was also tuned to occur over physiologically relevant O_2 pressures. Initial attempts to generate a murine cdb3 with an elevated deoxyHb affinity were guided by the mutations in human cdb3 that produced the undesirable excessive affinity. Surprisingly, deletion of neither of the murine sequences (del 1−10 and del 39−66) that corresponded with the human sequences (i.e., residues 1−10 and 29−52, respectively) whose deletion yielded a cdb3 with an extraordinarily high affinity for deoxyHb generated a murine cdb3 with an elevated affinity for deoxyHb. However, guided by the fact that the interaction between cdb3 and deoxyHb is electrostatic in nature, we hypothesized that replacement of some of the amino acids in sequence 25−31 with negative residues might generate a cdb3 with an increased affinity for deoxyHb (Figure 1B, subst 25−31). As seen in Figure 3C, binding of deoxyHb to the subst 25−31 mutant yielded the strongest fluorescence quenching yet observed with murine proteins, indicating that addition of these charged residues promotes an unusually high affinity for murine deoxyHb. The apparent absence of a similar sequence in nature suggests that higher cdb3−deoxyHb affinity is not functionally desirable.

Confirmation of the DeoxyHb Binding Site on Murine cdb3 by Analysis of the Equilibrium Dissociation Constant. Fluorescence resonance energy transfer is strongly dependent on the distance and orientation between the fluorescence donor and the associated quencher. For example, if the distance between donor and quencher were to decrease as a consequence of a mutation in cdb3, an increase in the level of quenching might be expected even in the absence of a change in affinity. To confirm that alterations in donor−quencher distance and/or orientation did not bias the data given above

for cdb3−deoxyHb relative affinity, we measured the equilibrium dissociation constants of the three most critical forms of cdb3 for murine deoxyHb: (i) wild-type cdb3, (ii) the cdb3 mutant with the lowest affinity for deoxyHb (subst 12−23), and (iii) the cdb3 mutant with the highest affinity for deoxyHb (subst 25−31). As shown in Figure 4, the cdb3

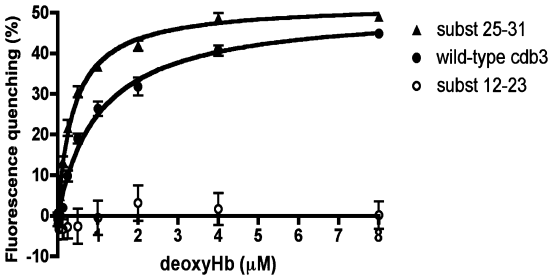


Figure 4. Evaluation of equilibrium binding affinity of deoxyHb for various isoforms of cdb3. Increasing concentrations of deoxyHb were incubated for 45 min at room temperature with cdb3-GFP or one of its mutants, and the equilibrium binding constant was calculated as described in Experimental Procedures (n = 3). Derived K_d values were 1.04 ± 0.14 μM for wild-type cdb3-GFP and 0.39 ± 0.03 μM for the sequence 25−31 substitution mutant. The sequence 12−23 substitution mutant displayed no measurable affinity for deoxyHb.

mutant that previously showed no affinity in the GFP quenching assay also showed no affinity in the equilibrium binding study. Moreover, the mutant that displayed the highest affinity for deoxyhemoglobin in the quenching assay also displayed the highest affinity in the equilibrium binding assay, lowering the equilibrium dissociation constant for deoxyhemoglobin from 1.04 μM (wild-type band 3) to 0.39 μM for the mutated band 3. These data confirm that the relative affinities of the three major forms of cdb3 for deoxyHb correspond to their relative extents of quenching upon addition of deoxyHb.

Confirmation of the DeoxyHb Binding Site on Murine cdb3 by Analysis of Hb−Oxygen Dissociation Curves. To confirm the deoxyHb binding site analyses described above, we determined the binding affinity of our various murine cdb3 mutants using a second and independent method. In this method, the ability of cdb3 to shift the oxygen dissociation curve for Hb to higher O_2 pressures is used as a measure of the affinity of cdb3 for deoxyHb.[3] Thus, the greater the shift in the P_{50} values of Hb (i.e., the O_2 pressure where Hb is half-saturated with oxygen) to higher O_2 pressures, the greater the affinity of the mutant cdb3 for deoxyHb.[3,38] In agreement with results from the FRET assay, the low-affinity mutant, subst 12−23, did not affect the O_2 dissociation curve of Hb but rather displayed the same P_{50} value as Hb alone (Figure 5). In contrast, the mutant that exhibited the greatest quenching capacity in the FRET assay also showed the largest right shift of the Hb−O_2 dissociation curve, confirming its exaggerated affinity for cdb3. As anticipated from the FRET data, removal of the first 10 amino acids from the NH_2 terminus of mouse cdb3 did not induce an elevated affinity for mouse Hb, i.e., in contradistinction to the impact of the same deletion in human cdb3.

DISCUSSION

Considerable effort has been devoted to characterizing the interaction between human deoxyHb and human band 3,[3,14,38−40] even to the point of mapping the amino acids

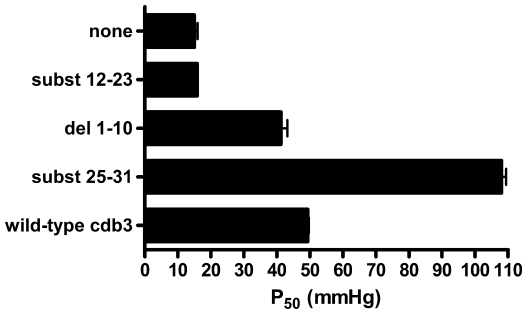


Figure 5. Confirmation of the deoxyHb binding site on murine cdb3 by evaluation of the effect of various NH_2-terminal mutants of cdb3 on the Hb−O_2 dissociation curve. Hb−O_2 binding curves were measured for Hb alone or Hb in the presence of wild-type cdb3, del 1−10, del 1−21, subst 12−23, or subst 25−31, and the affinity of deoxyHb for cdb3 was determined by analysis of the oxygen pressure at which the murine Hb became 50% saturated with O_2 (P_{50}).

involved in binding[3] and determining the crystal structure of a complex of deoxyHb with the NH_2 terminus of band 3.[38] The results collectively suggest that the NH_2 terminus of band 3 inserts into the 2,3-DPG binding cavity in the center of the deoxyHb tetramer, with residues 12−23 of band 3 establishing the major contact with amino acids along the walls of the deoxyHb cleft.[41,42] Because exposure to O_2 induces a "T" to "R" state transition that closes the central cationic cavity in deoxyHb, oxygenation of Hb leads to ejection of band 3 from the cavity, causing dissociation of oxyHb from the membrane.[38] We and others have hypothesized that this reversible association of deoxyHb with band 3 might directly or indirectly (via an induced conformational change in the anion transporter) alter the functional properties of proteins associated with band 3.[1,21,22] Because deoxyHb competes directly with glycolytic enzymes[19] and ankyrin (M. Stefanovic and P. Low, unpublished data) for binding to cdb3, a mechanism whereby changes in O_2 might modulate both erythrocyte metabolism and membrane structural properties can be readily envisioned. However, an explanation for how deoxyHb binding could regulate K^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransport, Na^+/H^+ antiport, or P_{sickle} is much more difficult to develop. While a variety of pathways can be proposed, we offer the following hypothesis as one that is supported by some experimental data. We propose that binding of deoxyHb to the cytoplasmic domain of band 3 induces a conformational change in the protein's membrane-spanning domain and that this conformational change is communicated to associated ion transporters via interfacial interactions. Evidence that significant communication occurs between the cytoplasmic and membrane-spanning domains of band 3 derives from studies showing that (i) association of Hb with cdb3 affects stilbene disulfonate binding to an externally exposed site on the membrane-spanning domain of band 3,[43,44] (ii) binding of Hb[45] or ankyrin[46] to cdb3 or phosphorylation of cdb3[47] alters the transport of anions through the membrane-spanning domain of the polypeptide, and (iii) binding of hemichrome to cdb3 induces binding of an autologous antibody to an external epitope on band 3.[48−50] If the conformational changes that mediate these functional alterations in the membrane-spanning domain of band 3 are also "sensed" by associated ion transporters, O_2 regulation of K^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransport, Na^+/H^+ antiport, and P_{sickle} might be envisioned.

dx.doi.org/10.1021/bi201623v | Biochemistry 2012, 51, 3264−3272

To evaluate whether the same O_2 regulatory mechanism might apply broadly across mammalian species, we explored whether the O_2-dependent Hb binding in humans might also occur in mice. On the basis of the alignment of the NH_2 termini of human and mouse cdb3s, the predicted murine Hb binding site for deoxyHb was predicted to reside between residues 21 and 33 (Figure 1A). However, when this sequence was deleted from murine cdb3, deoxyHb was found to display normal affinity for cdb3. In contrast, when residues 12−23 were mutated, Hb affinity was significantly compromised. Thus, although the binding site of Hb on murine cdb3 differs from that in humans, the ability of physiological changes in O_2 pressure to modulate Hb binding has been conserved. This suggests that O_2 modulation of red cell properties might constitute an important regulatory mechanism that is required for the adequate function of mammalian erythrocytes.

Curiously, mutations in human band 3 that generated a cdb3 with an abnormally high affinity for deoxyHb were also not conserved in the mouse. Thus, whereas deletion of residues 1−10 or 29−52 in human cdb3 promoted such affinity for deoxyHb that its dissociation could not be induced even at supraphysiological O_2 pressures, mutation of the same residues in the mouse had little effect on deoxyHb binding. In contrast, introduction of negatively charged residues directly adjacent to the Hb binding site on murine cdb3 was found to yield a cdb3 with greatly elevated Hb affinity, as demonstrated by both FRET and oxygen dissociation curve analysis (Figures 3C and 4, subst 25−31). Thus, the murine erythrocyte like the human erythrocyte appears to have a finely tuned O_2 regulatory system that responds to changes in O_2 pressure precisely over the physiological range of O_2 tensions.

Finally, the question of whether malfunction of this O_2-triggered switch in erythrocyte properties might have medical consequences naturally arises. That is, if a pathological condition or mutation were to cause sustained association of deoxyHb with band 3, would an observable "constitutively on" phenotype emerge? While there is currently insufficient information to provide an unequivocal answer to this question, several observations argue that it may ultimately be answered in the affirmative. Thus, a diversity of mutations in the central cavity of deoxyHb, where acidic amino acids are replaced with neutral or cationic residues, have been reported to lead to an activation of K^+/Cl^- cotransport.[51,52] If the consequent change in cdb3 affinity were to be communicated through the membrane-spanning domain of band 3 to the KCC, the change in K^+/Cl^- cotransport might be explained. Similarly, patients with mutant hemoglobins characterized by reduced O_2 affinities have been reported to have elevated 2,3-DPG levels in their erythrocytes.[53] While other explanations are again possible,[54] it is also conceivable that an increased level of binding of deoxyHb to cdb3 might lead to the constitutive displacement of glycolytic enzymes from band 3 and the consequent production of elevated levels of 2,3-DPG. Along the same lines, patients with compromised erythrocyte oxygenation due to poor gas exchange in the lungs (e.g., chronic obstructive pulmonary disease,[55] cystic fibrosis,[56] asthma,[57] and chronic cardiac insufficiency[58]) have been frequently reported to have erythrocytes with atypical deformabilities and/or morphologies. While other explanations are again possible, it should not be ignored that deoxyHb displacement of ankyrin from band 3 might contribute to these structural abnormalities. Finally, humans and dogs that have been exposed to high altitude/hypoxia are commonly observed to produce erythrocytes with prolonged filtration times.[59,60] Because the slower filtration of their erythrocytes can be reversed with pentoxifylline, a drug that regulates erythrocyte hydration through control of ion transporters,[61,62] it is plausible that the altered rheology of these individuals' red cells might derive from sustained deoxyHb−band 3 interactions leading to an abnormal ion balance.

Regardless of whether any of the pathologies described above are impacted by deoxyHb−band 3 interactions, the observation that an O_2 regulatory switch that controls these interactions is conserved in anucleate erythrocytes, despite considerable variability in the primary structure of band 3, suggests that the ability to regulate erythrocyte properties with O_2 is useful and that deviations from this, if they have accrued, appear to be inconsistent with life.

■ ASSOCIATED CONTENT

Supporting Information

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis and circular dichroism analysis show that mouse cdb3 and mutant proteins are pure and folded correctly, and the raw spectra of fluorescence quenching by deoxyHb are shown in Figure 3. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Address: 506 Oval Dr., 331B Wetherill Building, West Lafayette, IN 47907. Telephone: (765) 494-5273. Fax: (765) 494-5272. E-mail: plow@purdue.edu.

Funding

This work was supported by National Institutes of Health Grant GM24417-33.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Robert Franco and Mary Palascak for the assistance with the HEMOX instrument (Department of Internal Medicine, Division of Hematology-Oncology, University of Cincinnati, Cincinnati, OH). We greatly appreciate the gift of the GFPuv vector from Dr. David Thompson (Department of Chemistry, Purdue University). Also, we really appreciate the insights of Dr. Alan Rebar on this matter.

■ ABBREVIATIONS

cdb3, cytoplasmic domain of band 3; Hb, hemoglobin; deoxyHb, deoxyhemoglobin; GFP, green fluorescence protein; KCC, K^+/Cl^- cotransporter; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; 2,3-DPG, 2,3-diphosphoglycerate.

■ REFERENCES

(1) Barvitenko, N. N., Adragna, N. C., and Weber, R. E. (2005) Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell. Physiol. Biochem. 15*, 1−18.

(2) Castagnola, M., Messana, I., Sanna, M. T., and Giardina, B. (2010) Oxygen-linked modulation of erythrocyte metabolism: State of the art. *Blood Transfus. 8* (Suppl. 3), s53−s58.

(3) Chu, H., Breite, A., Ciraolo, P., Franco, R. S., and Low, P. S. (2008) Characterization of the deoxyhemoglobin binding site on human erythrocyte band 3: Implications for O_2 regulation of erythrocyte properties. *Blood 111*, 932−938.

(4) Hamasaki, N., Asakura, T., and Minakami, S. (1970) Effect of oxygen tension on glycolysis in human erythrocytes. *J. Biochem. 68*, 157−161.
(5) Rapoprot, I., Berger, H., Rapoport, S. M., Elsner, R., and Gerber, G. (1976) Response of the glycolysis of human erythrocytes to the transition from the oxygenated to the deoxygenated state at constant intracellular pH. *Biochim. Biophys. Acta 428*, 193−204.
(6) Kinoshita, A., Tsukada, K., Soga, T., Hishiki, T., Ueno, Y., Nakayama, Y., Tomita, M., and Suematsu, M. (2007) Roles of hemoglobin Allostery in hypoxia-induced metabolic alterations in erythrocytes: Simulation and its verification by metabolome analysis. *J. Biol. Chem. 282*, 10731−10741.
(7) Lewis, I. A., Campanella, M. E., Markley, J. L., and Low, P. S. (2009) Role of band 3 in regulating metabolic flux of red blood cells. *Proc. Natl. Acad. Sci. U.S.A. 106*, 18515−18520.
(8) Messana, I., Orlando, M., Cassiano, L., Pennacchietti, L., Zuppi, C., Castagnola, M., and Giardina, B. (1996) Human erythrocyte metabolism is modulated by the O_2-linked transition of hemoglobin. *FEBS Lett. 390*, 25−28.
(9) Giardina, B., Messana, I., Scatena, R., and Castagnola, M. (1995) The multiple functions of hemoglobin. *Crit. Rev. Biochem. Mol. Biol. 30*, 165−196.
(10) Chu, H., and Low, P. S. (2006) Mapping of glycolytic enzyme-binding sites on human erythrocyte band 3. *Biochem. J. 400*, 143−151.
(11) Higashi, T., Richards, C. S., and Uyeda, K. (1979) The interaction of phosphofructokinase with erythrocyte membranes. *J. Biol. Chem. 254*, 9542−9550.
(12) Murthy, S. N., Liu, T., Kaul, R. K., Kohler, H., and Steck, T. L. (1981) The aldolase-binding site of the human erythrocyte membrane is at the NH_2 terminus of band 3. *J. Biol. Chem. 256*, 11203−11208.
(13) Tsai, I. H., Murthy, S. N., and Steck, T. L. (1982) Effect of red cell membrane binding on the catalytic activity of glyceraldehyde-3-phosphate dehydrogenase. *J. Biol. Chem. 257*, 1438−1442.
(14) Low, P. S. (1986) Structure and function of the cytoplasmic domain of band 3: Center of erythrocyte membrane-peripheral protein interactions. *Biochim. Biophys. Acta 864*, 145−167.
(15) Campanella, M. E., Chu, H., and Low, P. S. (2005) Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane. *Proc. Natl. Acad. Sci. U.S.A. 102*, 2402−2407.
(16) Campanella, M. E., Chu, H., Wandersee, N. J., Peters, L. L., Mohandas, N., Gilligan, D. M., and Low, P. S. (2008) Characterization of glycolytic enzyme interactions with murine erythrocyte membranes in wild-type and membrane protein knockout mice. *Blood 112*, 3900−3906.
(17) Harrison, M. L., Rathinavelu, P., Arese, P., Geahlen, R. L., and Low, P. S. (1991) Role of band 3 tyrosine phosphorylation in the regulation of erythrocyte glycolysis. *J. Biol. Chem. 266*, 4106−4111.
(18) Low, P. S., Allen, D. P., Zioncheck, T. F., Chari, P., Willardson, B. M., Geahlen, R. L., and Harrison, M. L. (1987) Tyrosine phosphorylation of band 3 inhibits peripheral protein binding. *J. Biol. Chem. 262*, 4592−4596.
(19) Low, P. S., Rathinavelu, P., and Harrison, M. L. (1993) Regulation of glycolysis via reversible enzyme binding to the membrane protein, band 3. *J. Biol. Chem. 268*, 14627−14631.
(20) Cossins, A. R., and Gibson, J. S. (1997) Volume-sensitive transport systems and volume homeostasis in vertebrate red blood cells. *J. Exp. Biol. 200*, 343−352.
(21) Ellory, J. C., Gibson, J. S., and Stewart, G. W. (1998) Pathophysiology of abnormal cell volume in human red cells. *Contrib. Nephrol. 123*, 220−239.
(22) Gibson, J. S., Cossins, A. R., and Ellory, J. C. (2000) Oxygen-sensitive membrane transporters in vertebrate red cells. *J. Exp. Biol. 203*, 1395−1407.
(23) Drew, C., Ball, V., Robinson, H., Clive Ellory, J., and Gibson, J. S. (2004) Oxygen sensitivity of red cell membrane transporters revisited. *Bioelectrochemistry 62*, 153−158.
(24) Khan, A. I., Drew, C., Ball, S. E., Ball, V., Ellory, J. C., and Gibson, J. S. (2004) Oxygen dependence of K^+-Cl^- cotransport in human red cell ghosts and sickle cells. *Bioelectrochemistry 62*, 141−146.
(25) Muzyamba, M. C., Speake, P. F., and Gibson, J. S. (2000) Oxidants and regulation of K^+-Cl^- cotransport in equine red blood cells. *Am. J. Physiol. 279*, C981−C989.
(26) Speake, P. F., and Gibson, J. S. (1997) Urea-stimulated K-Cl cotransport in equine red blood cells. *Pfluegers Arch. 434*, 104−112.
(27) Gibson, J. S., and Ellory, J. C. (2003) *Red cell membrane transport in health and disease*, 1st ed., Springer-Verlag, Berlin.
(28) Gibson, J. S., Khan, A., Speake, P. F., and Ellory, J. C. (2001) O_2 dependence of K^+ transport in sickle cells: The effect of different cell populations and the substituted benzaldehyde 12C79. *FASEB J. 15*, 823−832.
(29) Motais, R., Garcia-Romeu, F., and Borgese, F. (1987) The control of Na^+/H^+ exchange by molecular oxygen in trout erythrocytes. A possible role of hemoglobin as a transducer. *J. Gen. Physiol. 90*, 197−207.
(30) Muzyamba, M. C., Cossins, A. R., and Gibson, J. S. (1999) Regulation of Na^+-K^+-$2Cl^-$ cotransport in turkey red cells: The role of oxygen tension and protein phosphorylation. *J. Physiol. 517* (Part 2), 421−429.
(31) Bennett, V., and Gilligan, D. M. (1993) The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. *Annu. Rev. Cell Biol. 9*, 27−66.
(32) Chang, S. H., and Low, P. S. (2003) Identification of a critical ankyrin-binding loop on the cytoplasmic domain of erythrocyte membrane band 3 by crystal structure analysis and site-directed mutagenesis. *J. Biol. Chem. 278*, 6879−6884.
(33) Jelkmann, W., and Baufer, C. (1976) What is the best method to remove 2,3-diphosphoglycerate from hemoglobin? *Anal. Biochem. 75*, 382−388.
(34) Riggs, A. (1965) Hemoglobin Polymerization in Mice. *Science 147*, 621−623.
(35) van Kampen, E. J., and Zijlstra, W. G. (1983) Spectrophotometry of hemoglobin and hemoglobin derivatives. *Adv. Clin. Chem. 23*, 199−257.
(36) Zhang, D., Kiyatkin, A., Bolin, J. T., and Low, P. S. (2000) Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. *Blood 96*, 2925−2933.
(37) Chetrite, G., and Cassoly, R. (1985) Affinity of hemoglobin for the cytoplasmic fragment of human erythrocyte membrane band 3. Equilibrium measurements at physiological pH using matrix-bound proteins: The effects of ionic strength, deoxygenation and 2,3-diphosphoglycerate. *J. Mol. Biol. 185*, 639−644.
(38) Walder, J. A., Chatterjee, R., Steck, T. L., Low, P. S., Musso, G. F., Kaiser, E. T., Rogers, P. H., and Arnone, A. (1984) The interaction of hemoglobin with the cytoplasmic domain of band 3 of the human erythrocyte membrane. *J. Biol. Chem. 259*, 10238−10246.
(39) Cassoly, R. (1983) Quantitative analysis of the association of human hemoglobin with the cytoplasmic fragment of band 3 protein. *J. Biol. Chem. 258*, 3859−3864.
(40) Weber, R. E., Voelter, W., Fago, A., Echner, H., Campanella, E., and Low, P. S. (2004) Modulation of red cell glycolysis: Interactions between vertebrate hemoglobins and cytoplasmic domains of band 3 red cell membrane proteins. *Am. J. Physiol. 287*, R454−R464.
(41) O'Donnell, S., Mandaro, R., Schuster, T. M., and Arnone, A. (1979) X-ray diffraction and solution studies of specifically carbamylated human hemoglobin A. Evidence for the location of a proton- and oxygen-linked chloride binding site at valine 1α. *J. Biol. Chem. 254*, 12204−12208.
(42) Richard, V., Dodson, G. G., and Mauguen, Y. (1993) Human deoxyhaemoglobin-2,3-diphosphoglycerate complex low-salt structure at 2.5 Å resolution. *J. Mol. Biol. 233*, 270−274.
(43) Salhany, J. M., and Cassoly, R. (1989) Kinetics of p-mercuribenzoate binding to sulfhydryl groups on the isolated cytoplasmic fragment of band 3 protein. Effect of hemoglobin binding on the conformation. *J. Biol. Chem. 264*, 1399−1404.
(44) Salhany, J. M., Cordes, K. A., and Gaines, E. D. (1980) Light-scattering measurements of hemoglobin binding to the erythrocyte

membrane. Evidence for transmembrane effects related to a disulfonic stilbene binding to band 3. *Biochemistry 19*, 1447−1454.
(45) Ducis, I., Kandrach, A., and Racker, E. (1988) Stimulation of $^{32}P_i$ transport into human erythrocyte ghosts and reconstituted vesicles by Mg^{2+} and hemoglobin. *J. Biol. Chem. 263*, 8544−8550.
(46) Kay, M. M., Bosman, G. J., and Lawrence, C. (1988) Functional topography of band 3: Specific structural alteration linked to functional aberrations in human erythrocytes. *Proc. Natl. Acad. Sci. U.S.A. 85*, 492−496.
(47) Bursaux, E., Hilly, M., Bluze, A., and Poyart, C. (1984) Organic phosphates modulate anion self-exchange across the human erythrocyte membrane. *Biochim. Biophys. Acta 777*, 253−260.
(48) Baggio, B., Bordin, L., Clari, G., Gambaro, G., and Moret, V. (1993) Functional correlation between the Ser/Thr-phosphorylation of band-3 and band-3-mediated transmembrane anion transport in human erythrocytes. *Biochim. Biophys. Acta 1148*, 157−160.
(49) Condon, M. R., Feketova, E., Machiedo, G. W., Deitch, E. A., and Spolarics, Z. (2007) Augmented erythrocyte band-3 phosphorylation in septic mice. *Biochim. Biophys. Acta 1772*, 580−586.
(50) Kannan, R., Labotka, R., and Low, P. S. (1988) Isolation and characterization of the hemichrome-stabilized membrane protein aggregates from sickle erythrocytes. Major site of autologous antibody binding. *J. Biol. Chem. 263*, 13766−13773.
(51) Nagel, R. L., Krishnamoorthy, R., Fattoum, S., Elion, J., Genard, N., Romero, J., and Fabry, M. E. (1999) The erythrocyte effects of haemoglobin O(ARAB). *Br. J. Haematol. 107*, 516−521.
(52) Olivieri, O., Vitoux, D., Galacteros, F., Bachir, D., Blouquit, Y., Beuzard, Y., and Brugnara, C. (1992) Hemoglobin variants and activity of the (K^+Cl^-) cotransport system in human erythrocytes. *Blood 79*, 793−797.
(53) Nagel, R. L. (2000) *Disorders of hemoglobin: Genetics, pathophysiology, and clinical management*, 1st ed., Cambridge University Press, Cambridge, U.K.
(54) Clench, J., Ferrell, R. E., and Schull, W. J. (1982) Effect of chronic altitude hypoxia on hematologic and glycolytic parameters. *Am. J. Physiol. 242*, R447−R451.
(55) Ahmad, B., Thieme, H., Bloch, W., Ferrari, N., Raabe-Oetker, A., and Brixius, K. (2011) Influence of acute and longtherm physical activity on erythrocyte flexibility in patients suffering from chronic obstructive pulmonary disease (COPD). *Acta Physiol. 201*, P314.
(56) Novak, Z., and Gyurkovits, K. (1990) Examination of red blood cell deformability in cystic fibrosis. *Acta Univ. Carol., Med. 36*, 68−70.
(57) Todoriko, L. D. (1998) [Changes in the morphofunctional state of the erythrocyte membranes in bronchial asthma in patients of different ages]. *Vrach. Delo.,*, 51−54.
(58) Romashchenko, O. V., Kamenev, V. F., Tret'iakov, A., and Alferov, P. K. (2009) [Erythrone system in aged patients with chronic cardiac insufficiency]. *Klin. Med. (Moscow, Russ. Fed.) 87*, 21−25.
(59) Chick, T. W., Scotto, P., Icenogle, M. V., Sikes, C. W., Doyle, M. P., Riedel, C. E., Wood, S. C., and Loeppky, J. A. (1988) Effects of pentoxifylline on pulmonary hemodynamics during acute hypoxia in anesthetized dogs. *Am. Rev. Respir. Dis. 137*, 1099−1103.
(60) Palareti, G., Coccheri, S., Poggi, M., Tricarico, M. G., Magelli, M., and Cavazzuti, F. (1984) Changes in the rheologic properties of blood after a high altitude expedition. *Angiology 35*, 451−458.
(61) Porsche, E., and Stefanovich, V. (1981) [The effect of pentoxifylline on the Ca^{2+}-induced potassium efflux and on the ATPase-activity of erythrocytes (author's transl)]. *Arzneimittelforschung 31*, 825−828.
(62) Stuart, J., Stone, P. C., Bilto, Y. Y., and Keidan, A. J. (1987) Oxpentifylline and cetiedil citrate improve deformability of dehydrated sickle cells. *J. Clin. Pathol. 40*, 1182−1186.