Modulation of red cell glycolysis: interactions between vertebrate hemoglobins and cytoplasmic domains of band 3 red cell membrane proteins

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Weber, Roy E., Wolfgang Voelter, Angela Fago, Hartmut Echner, Estela Campanella, and Philip S. Low. Modulation of red cell glycolysis: interactions between vertebrate hemoglobins and cytoplasmic domains of band 3 red cell membrane proteins. Am J Physiol Regul Integr Comp Physiol 287: R454–R464, 2004. First published April 15, 2004; 10.1152/ajpregu.00060.2004.—Several vital functions/physical characteristics of erythrocytes (including glycolysis, the pentose phosphate pathway, ion fluxes, and cellular deformability) display dependence on the state of hemoglobin oxygenation. The molecular mechanism proposed involves an interaction between deoxyhemoglobin and the cytoplasmic domain of the anion-exchange protein, band 3 (cdB3). Given that band 3 also binds to membrane proteins 4.1 and 4.2, several kinases, hemichromes, and integral membrane proteins, and at least three glycolytic enzymes, it has been suggested that the cdB3-deoxyhemoglobin interaction might modulate the pathways mediated by these associated proteins in an O2-dependent manner. We have investigated this mechanism by synthesizing 10-mer peptides corresponding to the NH2-terminal fragments of various vertebrate cdB3s, determining their effects on the oxygenation reactions of hemoglobins from the same and different species and examining binding of the glycolytic enzyme glycereraldehyde-3-phosphate dehydrogenase to the erythrocytic membrane of mouse erythrocytes. The cdB3 interaction is strongly dependent on pH and the number of negative and positive charges of the peptide and at the effector binding site, respectively. It lowers the O2 association equilibrium constant of the deoxygenated (Tense) state of the hemoglobin and is inhibited by magnesium ions, which neutralize cdB3’s charge and by 2,3-diphosphoglycerate, which competes for the cdB3-binding site. The interaction is stronger in humans (whose erythrocytes derive energy predominantly from glycolysis and exhibit higher buffering capacity) than in birds and ectothermic vertebrates (whose erythrocytes metabolize anaerobically and are poorly buffered) and is insignificant in fish, suggesting that its role in the regulation of red cell glycolysis increased with phylogenetic development in vertebrates.

allosteric interaction; erythrocyte; oxygen binding; glycolytic enzymes

THE RATES OF GLYCOLYSIS, the pentose phosphate pathway, and a number of ion transport pathways in vertebrate erythrocytes have all been shown to display a dependence on the state of Hb oxygenation. Thus, on Hb deoxygenation, glycolysis is reported to accelerate (12, 27, 31, 35), the pentose phosphate pathway is observed to decline (27), flux through the KCl cotransporter is seen to decrease (11), and the activities of the Na-K-2Cl cotransporter and the Na/H exchanger are found to rise (11). Consideration of the possible molecular mechanisms responsible for these O2-dependent changes has largely focused on the significantly higher affinity of deoxyhemoglobin (deoxyHb) than oxyhemoglobin (oxyHb) for the cytoplasmic domain of band 3, the anion-exchange protein (AE1) and most abundant polypeptide of the erythrocyte membrane (25). Thus, in addition to binding ankyrin, protein 4.1, protein 4.2, several kinases, hemichromes, a number of integral membrane proteins, and at least three glycolytic enzymes (3, 13, 25, 26), the cytoplasmic domain of band 3 (cdB3) is also believed to preferentially associate with the deoxygenated state of Hb (6, 42). In fact, crystallographic data demonstrate that the NH2-terminal 11 residues of human erythrocyte band 3 extend ~18Å into the central 2,3-diphosphoglycerate (DPG) binding cleft of human deoxyHb, occupying a cavity that becomes occluded on Hb oxygenation (42). Consistent with these structural data, human cdB3 decreases the affinity of Hb for O2, and binding competition assays demonstrate that human cdB3 competes with DPG for occupancy of the central cleft in the deoxyHb tetramer (6, 42). Based on this selectivity of cdB3 for deoxyHb and the established role of band 3 as a major organizing center of the erythrocyte membrane (3, 13, 25, 26), the hypothesis has been offered that reversible association of deoxyHb with the cdB3 might alter the interaction of the entire band 3 polypeptide with associated proteins, thereby modulating the pathways mediated by these proteins in an O2-dependent manner (11, 27).

Assuming that evolution of a regulatory switch that can control erythrocyte functions in an O2-dependent manner is desirable, the question naturally arises whether nonhuman vertebrate Hbs might also have evolved an O2-dependent affinity for their respective cdB3s. Arguments against such a hypothesis include the fact that the NH2 termini of the vertebrate band 3s sequenced to date are not highly conserved (see protein sequence databases) and the findings that the NH2 terminus of trout band 3 has no tangible effects on the affinity of isolated trout Hb components for O2 (21, 47). Arguments in support of this contention rely primarily on the observations that the NH2 termini of all known cdB3s are polyamnic in nature and that the deoxygenated states of most vertebrate Hbs display high affinity for polyanions such as DPG, inositol pentaphosphate (IPP), ATP, or GTP. Thus, assuming that the polyamnic NH2 termini of nonhuman cdB3s can also compete...
for the cationic central cavities in their respective deoxyHbs, a similar O$_2$-dependent regulatory switch might be anticipated in other vertebrate erythrocytes.

We have undertaken to investigate the Hb-band 3 interaction in humans as well as in lower vertebrates by synthesizing the 10-mer peptides corresponding to the NH$_2$-terminal segments of their cdB3s and examining the effects of these peptides on the O$_2$-binding properties of Hbs from the same and different species. In contrast to mitochondria-free human erythrocytes that derive energy predominantly from anaerobic metabolism, bird and ectothermic vertebrate erythrocytes are richly supplied with mitochondria and rely predominantly on aerobic metabolism (44, 54). As a fish model, we have used catfish (Hoplolamprostum littorale) cathodic Hb$^{\text{c-a}}$, which shows pronounced phosphate sensitivity, in contrast to the cathodic counterpart (Hbl) from trout. Also, it lacks the negatively charged residue (Asp at position NA2 of the $\beta$-chains) (48) that is considered to hinder peptide binding in trout Hb. Bird studies focused on the major chicken isohemoglobin (isoHb), HbA, and the minor component, HbD, where enhanced cooperativity at high levels of O$_2$ saturation indicate self-association of the tetramers (24).

The $\alpha$-chains of bird Hbs A and D, moreover, show marked homologies to those of turtle Hbs A and D (38). For comparative measurements in amphibians, we used Hb from the high-altitude Andean frog Telmatobius peruvianus.

We further examined the interactive effects of pH, of organic phosphate effectors that may compete with cdB3 for binding to Hb, and of Mg$^{2+}$ that may complex with anionic cdB3 to decrease its allosteric interaction with Hb; investigated the allosteric control mechanism underlying the marked cdB3 sensitivities of human and chicken Hbs; and carried out microscopic investigations demonstrating membrane localization of the glycolytic enzyme GAPDH in intact nonhuman red cells.

**EXPERIMENTAL PROCEDURES**

**Hb preparation.** Hemolysates from the Amazonian catfish $H$. littorale, chickens, and a nonsmoking adult human were prepared from washed cells as previously described (48). Cathodic $H$. littorale isoHb (HbC) was isolated by ion-exchange chromatography on a 27 $\times$ 2 cm column of DEAE-Sephasel gel, eluted in a 0 to 0.1 M NaCl gradient as earlier detailed (48), and concentrated by centrifugation in Centrifi prep-10 tube-filters (cut-off 10,000; Amicon). The major isoHb (Hbl) of $T$. peruvianus was prepared as previously described (53). Chicken Hbs A and D were isolated by preparative isoelectric focusing at 4°C on a 440 ml LKB preparative column containing 0.45% C on a 440 ml LKB preparative column containing 0.45% 

**Peptide synthesis.** We synthesized 10-mer peptides corresponding to NH$_2$-terminal sections of band 3 proteins of trout, chicken, and humans. The primary structures are Met-Glu-Asn-Asp-Leu-Ser-Phe-Gly-Glu-Asp (trout) (14) (Swiss-Prot P32847), Met-Glu-Gly-Pro-Gly-Gln-Asp-Thr-Glu-Asp (chicken) (23) (Swiss Prot P15755), and Met-Glu-Glu-Leu-Gln-Asp-Tyr-Glu-Asp (human) (22) (Swiss Prot P02730).

The three NH$_2$-terminal fragments of band 3 proteins were synthesized on an Eppendorf ECOSYN P solid-phase peptide synthesizer (Hamburg, Germany) employing a 9-fluorenylmethoxycarbonyl (Fmoc)-Asp(OtBu) S parahydroxybenzyl resin (Rapp Polymere, Tübingen, Germany; Refs. 1, 2). All amino acids were incorporated with the $\alpha$-amino functions protected by the Fmoc group. Side chain functionalities were protected as tert-buty1 esters (asparatic acid, glutamic acid), tert-buty1 ethers (serine, threonine, tyrosine), and trityl derivatives (asparagine). Coupling was performed using a fourfold excess in protected amino acids and the coupling reagent 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate plus 1.2 ml 12.5% disopropylethylamine dissolved in N,N-dimethyl formamide (DMF) over the resin loading. Before coupling the protected amino acids, the Fmoc groups were removed from the last amino acid of the growing fragment using 25% piperidine in DMF. After cleavage of the NH$_2$-terminal Fmoc group, the peptide was removed from the resin under simultaneous cleavage of the amino acid side chain protecting groups by incubation in a mixture of trifluoroacetic acid (TFA; 12 ml), ethanedithiol (0.6 ml), thioanisole (0.3 ml), anisole (0.3 ml), water (0.3 ml), and triisopropylsilane (0.1 ml) for 3 h. The mixture was filtered and washed with TFA, and the combined filtrates were precipitated with anhydrous diethyl ether. The crude products were further purified by HPLC on a Nucleosil 100 C$_18$ (7 $\mu$m) 250 $\times$ 20 mm column, monitoring the elution at 214 nm. The peptides were assayed for purity (>98%) by analytical HPLC, amino acid analyses, and matrix-assisted laser desorption ionization mass spectrometry.

**$O_2$ equilibrium studies.** $O_2$ equilibrium of ultrathin layers of Hb solutions were measured using a modified $O_2$ diffusion chamber, by continuously recording absorption during stepwise increases in $O_2$ tensions of equilibrating gases. Gas mixtures were prepared using cascaded Wösthoff gas mixing pumps supplied with pure (99.998% N$_2$ and air or O$_2$; 45, 46). Half-saturation $O_2$ tensions ($P_{50}$) and the Hill cooperativity coefficients at $P_{50}$ ($n_{so}$) were interpolated from

$$\log [S/(1 - S)] \text{ vs. } \log P_{50} \text{ plots, where } S \text{ is the fractional saturation.}$$

The method shows high reproducibility ($P_{50} = 4.73 \pm 0.04, N = 6$, for human Hb at 25°C and pH 7.4) (46). Chloride was added as KCl and assayed using a Radiometer CMT10 titrator. pH values were measured using a micro pH electrode (type G 299), a BMS2 Mk 2 apparatus, and a pHM 64 meter (Radiometer, Copenhagen, Denmark). $O_2$ equilibrium was measured near different target pH values (e.g., 7.5, 7.0, and 6.5) in the presence of 0.1 M HEPES buffers. The $P_{50}$ values at the exact pH values were then interpolated from log $P_{50}$ vs. pH regressions. The effects of Mg$^{2+}$ on cdB3-Hb interaction were investigated by addition of MgCl$_2$. Opposite effects of this salt on Hb-$O_2$ affinity in the absence and presence of the peptide permitted distinction between the effects of Mg$^{2+}$ and Cl$^-$ (see DISCUSSION).
Glycolytic enzyme binding. Binding of GAPDH in mouse red cells was evaluated by removing the erythrocytes via periorbital bleeding and washing the cells in isotonic PBS containing 5 mM glucose (330 mosM) to remove the serum and buffy coat. Erythrocytes were then fixed in PBS buffer containing 0.5% acrolein for 5 min and washed 3× in PBS buffer containing 0.1 M glycine (PBS-glycine) to remove the detergent. Cells were then blocked with PBS-glycine containing 0.2% gelatin and incubated with rabbit anti-human GAPDH diluted 1:100 in the blocking solution. After washing in blocking solution to remove unbound primary antibody, cells were stained with Rhodamine-Red-X-conjugated affinity-purified donkey anti-rabbit IgG that was preabsorbed to mouse IgG (Jackson ImmunoResearch). Erythrocytes were washed again in blocking solution, mounted on polylysine-coated slides, and viewed by confocal microscopy using a Bio-Rad MRC 1024 on a Nikon Diaphot 300 microscope.

RESULTS

All cdB3 peptides were found to interact with the different Hbs investigated but with greatly varying effects. At pH 7.0, the O₂ affinity of catfish (H. littorale) Hb shows only slight sensitivity to the peptides, compared with the pronounced effect of 0.1 M chloride (Fig. 2, A and C). At lower pH (6.5), however, trout and chicken peptides markedly reduce O₂ affinity, exerting a similar effect to chloride ions (Δlog P₅₀ ~ 0.16), whereas human peptide induces an even greater shift (Δlog P₅₀ ~ 0.31) that, however, remains less than that (Δlog P₅₀ ~ 0.52) of the native phosphate modulator ATP (Fig. 2, B and D). Andean frog Hb shows a similarly low peptide sensitivity (Δlog P₅₀ ~ 0.016 and ~0.032 at pH 6.8, for trout and human peptides, respectively; Fig. 3).

Chicken HbA exhibits a considerably higher peptide sensitivity than that of the ectothermic vertebrate Hbs; at 7.6 and 7.0, Δlog P₅₀ shifts induced by chicken peptide are 0.20 and 0.44, respectively (Fig. 4, Aa and Ac). Again, human peptide induces a much larger effect (Δlog P₅₀ ~ 0.68 at pH 7.0), which, however, is only about half of that (Δlog P₅₀ ~ 1.36) induced by inositol hexaphosphate (IHP; a structural analog of IPP found in bird erythrocytes). The peptide and IHP sensitivities of chicken HbD (Δlog P₅₀ ~ 0.42 and 1.0 at pH 7.0; Fig. 4B) are similar to those of chicken HbA, despite the overall higher O₂ affinities in HbD.

Human Hb shows the largest peptide effects (at pH 7.0, Δlog P₅₀ ~ 0.29 for chicken and trout peptides and ~0.57 for the human peptide). The effect of human peptide exceeds that of 0.1 M chloride and of DPG at a DPG/Hb(tetramer) ratio of

Fig. 2. A and B: semilogarithmic plots of O₂ equilibria of catfish (Hoplosternum littorale) HbCa measured in 0.1 M HEPES buffer at pH 6.98 (A) and 6.54 (B) in the absence of added effectors (C) and in the presence of 10-mer peptides (P) corresponding to the NH₂ termini of the cdB3 from trout (●), chickens (●), and humans (●) or in the presence of ATP (●) or ATP + trout peptide (●). C and D: histograms showing the effects of the peptides on interpolated half-saturation O₂ tension (P₅₀) values at pH 7.0 and pH 6.5 in the absence (str) and presence of peptides, Cl, and ATP (as indicated). Experimental conditions: temperature, 25°C; buffer, 0.1 M HEPES; [heme], 0.31 mM; peptide/tetrameric Hb ratio, 5; ATP/Hb ratio (where present), 1.5; [chloride] (where present), 0.10 mM. Shaded areas indicate peptide-induced shifts in P₅₀.
0.5 (Δlog P50 ~ 0.44 and ~0.38, respectively) and equals that of DPG/Hb, 1.5 (0.61) (Fig. 5C). Also the human peptide effects persist in the presence of 0.1 M chloride (Δlog ~0.14) and at a DPG/Hb ratio of 1.5 (Δlog ~0.21).

The increasing sensitivities of fish, chicken, and human Hbs to fish, chicken, and human peptides, respectively, is reflected in the observations that, in the presence of organic phosphates, cdB3 increases O2 affinity of fish Hb (Fig. 2D), has no effect on bird HbA (Fig. 4Ac), and further decreases that of human Hb (Fig. 5C). These observations emphasize the need to take into account interactive effects of combinations of different ligands.

The peptides markedly increase the Bohr factors (Δlog P50/ΔpH) of the Hbs, reflecting increased protonation of the binding sites at low pH, but they have no significant effect on cooperativity under the conditions of the measurement (Figs. 3 and 6). Strikingly, 7 mM MgCl2 increases the Bohr factor in stripped human Hb (from 0.30 to 0.48) but decreases it in the presence of human peptide (from −0.30 to −0.48) but decreases it from −1.08 to −0.74; see Fig. 6).

Fig. 3. O2 equilibria of frog (Telmatobius peruvianus) HbII in the absence (strain) and presence of trout and human peptides at 20° and pH 6.8. Inset: pH dependence of the peptide effects.

Fig. 4. A: O2 equilibria of chicken HbA in the absence (strain) and presence of trout, chicken, and human cdB3 peptides at pH ~7.61 (a) and ~7.02 (b) and the P50 shifts induced by the peptides and inositol hexaphosphate (IHP) at pH 7.0 (c). [Heme], 0.28 mM. Other conditions as in Fig. 2. B: effects of 10-mer chicken cdB3-peptide and IHP, singly and in combination on chicken HbD at pH ~7.59 (a) and ~7.02 (b), and the effector-induced O2 affinity shifts at pH 7.0 (c). Other conditions as in Fig. 2.
Fig. 5. Effects of 10-mer trout, chicken, and human peptides in the absence and presence of 2,3-diphosphoglycerate (DPG) (at DPG/Hb tetramer ratios of 0.5 and 1.5) on O2 equilibria of human Hb at pH 7.58 (A) and 6.97 (B) and the effector P50 shifts at pH 7.0 (C). [Heme], 0.45 mM. Other conditions as in Fig. 2.

Fig. 6. Influences of chicken and human peptides on Bohr effect and cooperativity coefficient at half-saturation (n50) of chicken HbA and HbD (A) and human Hb (B) in the absence (open symbols) and presence (filled symbols) of chicken and human peptides. The numbers on the linear regression lines indicate the Bohr factors (Δlog P50/ΔpH).
The observations that divalent ions like Mg$^{2+}$ complex with organic phosphates from human and fish erythrocytes (DPG and ATP/GTP, respectively) to decrease their allosteric potencies (4, 52) prompted investigation of the effects of Mg$^{2+}$ on the modulator potency of cdB3. As shown in Fig. 7, in the presence of peptide at pH 7.0, the $O_2$ affinity of human Hb gradually increases with increasing MgCl$_2$ concentration, whereas the salt has the opposite effect in the absence of peptide (which is attributable to increased chloride concentrations). The specific effect of the peptide (indicated by the difference between the open and closed symbols in Fig. 7) thus decreases with increasing Mg$^{2+}$ concentration but remains distinct at high pH (Fig. 7A) despite the concomitant reduction in the sensitivity to Mg$^{2+}$ in the presence of the peptide.

As illustrated for human Hb (Fig. 8), the maximum DPG-induced affinity change is much larger in the absence than in the presence of the peptide ($\Delta \sim -0.6$ and $\sim -0.15$, respectively). Thus the peptide potently decreases the DPG sensitivity of human Hb, whereby its own effect decreases with increasing DPG/Hb ratio. In the additional presence of MgCl$_2$ that neutralizes cdB3’s charge, the peptide effect is further reduced (Fig. 8).

Extended Hill plots, constructed to elucidate the allosteric control mechanism, show that chicken as well as human peptides reduce $O_2$ affinity of the corresponding Hbs by decreasing the $O_2$ association equilibrium constant of Hb in the deoxygenated state, $K_T$, without markedly changing that of the oxygenated state, $K_R$ (Fig. 9). Estimated from the y-intercept of the asymptotes of the upper and lower parts of the curves at log $P_{O_2} = 0$, the $K_T$ and $K_R$ values for stripped chicken Hb and peptide are 0.096 and 1.41 mmHg$^{-1}$, respectively. The $K_R/K_T$ ratio indicates a 15-fold higher $O_2$ affinity in the oxygenated compared with the deoxygenated state. In the presence of the peptide, the $K_T$ value decreases to $\sim 0.0276$ mmHg$^{-1}$. This increases $K_R/K_T$ to $\sim 51$ and raises the free energy of heme-hemoglobin interaction, $\Delta G = RT \ln (K_R/K_T)$, where R is the gas constant and $T$ is the absolute temperature, from $\sim -6.5$ to $\sim -9.6$ kJ/mol. Qualitatively similar effects are illustrated for human Hb (Fig. 9B).

Because $O_2$-dependent binding of Hb to band 3 takes on major significance only as a mechanism of modulating other band 3-protein interactions, we decided to evaluate whether proteins believed to bind the human erythrocyte membrane in an $O_2$-dependent manner might also associate with the membrane in a nonhuman vertebrate red cell. GAPDH probably constitutes the best test of this hypothesis, because it is well established to bind band 3 in the human erythrocyte membrane and because its binding site on band 3 directly overlaps the binding site of deoxyHb (25, 42), resulting in $O_2$-dependent binding in fresh intact human red cells (Campanella and Low, unpublished observations). To begin to evaluate this issue, fresh murine erythrocytes were fixed and stained with an antibody specific for GAPDH. As shown in the confocal micrographs of Fig. 10, application of secondary antibody alone yields no membrane staining (Fig. 10, A and B), whereas incubation with rabbit anti-GAPDH, followed by staining with donkey anti-rabbit IgG results in selective membrane labeling (Fig. 10, C and D).

**DISCUSSION**

$O_2$-dependent cdB3-Hb interaction in vertebrates. We demonstrate that 10-mer peptides corresponding to the NH$_2$ termini of trout, chicken, and human cdB3 undergo oxygenation-linked binding to catfish Hb$^{Ca}$, Andean frog Hb, human Hb, and avian...
Hbs A and D [that show homology with turtle Hbs A and D (38) (Figs. 2–5)], suggesting that this interaction is ubiquitous in vertebrate animals. However, the magnitudes of the interactions, and consequently their physiological relevance, differ substantially among different species. As shown in Fig. 11, the cdB3-Hb interaction is small between fish Hb and fish peptide, larger between bird Hb and bird peptide, and largest between human Hb and human peptide, even though chicken Hb shows greater cdB3 sensitivity than human Hb to all peptides (Fig. 11), which correlates with the involvement of two additional amino acid residues in IPP binding to bird Hbs than is the case for DPG binding to human Hb (34).

Inasmuch as the deoxygenated forms of these Hbs bind to cdB3 at the inner surfaces of their red cell membranes in competition with glycolytic enzymes (25) and other proteins, they thus may play a role as sensors linking erythrocyte functions to \( \text{O}_2 \) availability. Our visualization of GAPDH binding to mouse erythrocyte membranes (Fig. 10) confirms this and shows that this interaction is not limited to human erythrocytes. It obviously remains to be determined whether GAPDH in the other vertebrate species studied in this report are similarly membrane localized.

The effects of anionic cdB3 peptides on Hb-\( \text{O}_2 \) affinity invariably increased with declining pH (compare A and B of Figs. 2, 4, and 5). The observation that the effect is considerably larger for human 10-mer peptide, which contains six negatively charged amino acid residues, than for trout and chicken peptides, which only have four, favors prediction of cdB3-Hb interactions in other species. This correlation furthermore shows that 10-mer peptides are sufficiently large to investigate cdB3-Hb interactions. A similar inference derives from interactions of 15-mer human peptides with human HbS that indicate that only 5–7 residues extend internally in the DPG receptor locus of the Hb molecules, while the remaining 10–8 residues project externally and inhibit polymerization by steric hindrance (7).

X-ray crystallography (42) shows that the Hb binding site for an 11-mer human cdB3 peptide includes Arg 104 of both \( \beta \)-chains, as well as most of the basic residues within the DPG binding site between the \( \beta \)-chains, i.e., comprising Val-NA1(\( \beta \)1), His-NA2 (\( \beta \)2), Lys-EF6(\( \beta \)82), and His-H21(\( \beta \)143). Changes at these sites predictably exert marked effects. The reaction of cyanate with the \( \alpha \)-amino Val (NA1) residues of the \( \alpha \)- and \( \beta \)-chains of human Hb dramatically decreases binding.

Fig. 9. Extended Hill plots of chicken Hb at pH 7.02 (A) and human Hb at pH 7.0 (B), in the absence (○) and presence (■) of chicken (A) and human (B) peptides. The affinity constants (K) of the deoxygenated (T) and oxygenated (R) states can be estimated from \( \gamma \)-intercepts at \( \log \text{PO}_2 = 0 \) of extrapolated lower and upper asymptotes of the Hill plots. Heme concentrations 0.29 mM (chicken Hb) and 0.46 mM (human Hb).

Fig. 10. Confocal microscope image of GAPDH staining in mouse red blood cells (RBCs). Micrographs B and D are direct bright-field images of the cells shown in micrographs A and C. The sample in micrograph C is stained first with rabbit antibody to GAPDH and then rhodamine red X-labeled goat anti-rabbit IgG, whereas the sample in micrograph A is stained only with the rhodamine red X-labeled donkey anti-rabbit IgG as a control.
to band 3 sites (8). Studies of cdB3 interactions of fetal and adult Hbs that show substitutions at the phosphate binding sites are in progress.

The distinct (albeit small) effect of peptides on O₂ affinity of catfish Hb compares contrasts with the insensitivity of cationic and anodic Hbs to 10-mer and 20-mer trout cdB3 peptides, which correlates with negatively charged Asp and Glu residues found in trout HbI and HbIV, respectively, at NA2, (21, 47). In this light, the distinct response of cdB3 is attributable to positively charged His at NA2, (48). These correlations suggest that the O₂ affinities of teleost fish Hbs (like carp isoHbs and anodic and cationic eel Hbs) that have negatively charged NA2, Glu residues will not exhibit significant cdB3 sensitivity. Indeed, Hb of the anguillid, *Symenchesia parasitica*, from deep sea hydrothermal-vent habitats shows no sensitivity to trout cdB3 at pH 7.0 (49).

The presence of cdB3 effects in chicken Hbs A and D indicates that both isoHbs commonly encountered in birds may be implicated in the regulation of glycolysis in bird red cells and is consistent with the fact that these isoHbs differ only in their α-chains and have identical β-chains that harbor the DPG and cdB3 binding locus (24). The cdB3 sensitivity of chicken HbD moreover indicates that self-association of tetramers does not preclude access to the phosphate binding site, as is suggested by the fact that IHP aids in the formation of highly cooperative octamers (24).

**Allosteric control mechanism.** In decreasing *K*ₐ without markedly affecting the *K*ₐ values of the Hb molecules, the allosteric mechanism associated with peptide binding is homologous to that of DPG in human Hb and of the nucleoside triphosphates, ATP and GTP, at comparable phosphate/Hb ratios in fish Hb (50). As with the phosphate effectors, cdB3 accordingly raises both the free energy of heme- heme interactions and cooperativity. Calculated as *ΔG*ₐ = RT ln (*K*ₐ,cdB3/*K*ₐ,str), where *K*ₐ,cdB3 and *K*ₐ,str are values in the presence and absence of cdB3, respectively, the T-state energy differences associated with peptide binding are approximately 3.1 and 2.7 kJ·mol⁻¹·O₂⁻¹ for chicken and human Hbs at pH 7.0 (Fig. 9, A and B), which correspond broadly with the value of 3.8 kJ·mol⁻¹·O₂⁻¹ calculated from data on DPG binding to human Hb at pH 7.3 (51).

**Influences of other cytosolic components.** To discern the in vivo significance, the impact of other ionic factors from the cytosolic scenery needed to be taken into account. Previous studies have shown that the interaction between human Hb and isolated cdB3 fragments is abolished on addition of GAPDH (5) and that DPG as well as NaCl dissociates the cdB3-Hb complex (6). Apart from organic phosphates (that compete with cdB3 for the stereochemically complementary locus between the β-chains), divalent cations may perturb cdB3-Hb interactions by complexing with anionic phosphates. We find that whereas increasing MgCl₂ concentrations decrease the O₂ affinity of the stripped human Hb (as expected with increasing chloride ion concentration), they increase Hb-O₂ affinity in the presence of the peptide (Fig. 7). This reveals that the action of MgCl₂ in decreasing the net anionic charge of the peptides (and their allosteric effectiveness) overrides the effect of the chloride counterions. However, a pronounced peptide effect (*ΔlogP*ₐ₀ ~0.14 and ~0.38, respectively, at pH 7.5 and 7.0; Fig. 7) persists in the presence of 4.6 mM MgCl₂ concentration recorded for human red cells (15). In life the cation-induced reduction may be much less, given that the concentrations of free MgCl₂ in oxygenated and deoxygenated human red cells appear to be only 13 and 21%, respectively, of the total magnesium concentration found in red cells (30).

We show that the peptide effect decreases with increasing DPG/Hb ratio in the absence of MgCl₂ and that this effect is further reduced in the presence of MgCl₂ (Fig. 8). The potentiation of the Bohr effects by MgCl₂ or peptides (Fig. 6) is analogous to the effects of chloride and organic phosphate anions in vertebrate Hbs. The increased Bohr effect of Hb in the presence of peptide and MgCl₂ (compared with the value with peptide only) thus indicates neutralization of peptide charge by MgCl₂.

**Functional significance.** A key question is to what extent the in vitro data can be extrapolated to the in vivo situation. The in vitro cdB3 sensitivities of the Hbs studied are consistent with the view that the deoxygenated (T state) Hb molecules displace glycolytic enzymes from the cytoplasmic tails of band 3 at low O₂ tension, thereby activating glycolysis. As shown earlier, glycolysis can be increased over 30-fold by experimentally lowering the availability of these enzyme binding sites (26). The Hb-cdB3 interaction is much greater in mammalian erythrocytes, which are almost wholly dependent on glycolytic energy metabolism, than in fish and bird erythrocytes, which have predominantly aerobic metabolism. The low peptide sensitivities of fish Hbs argue against a tangible transducer role of fish Hb, unless another site exists where Hb and glycolytic enzymes compete for binding. However, the strong dependence of cation fluxes in turkey erythrocytes on Hb oxygenation (32) argues that Hb must retain some level of transducer function in avian species.

The pronounced difference in the magnitude of the cdB3-Hb interaction (large in humans and small in eutherian vertebrates) may be related to the differences in cellular buffering capacity, which is high in mammals and small in fish (16). Thus in contrast to the large pH variations occurring in fish erythrocytes, those in mammalian erythrocytes may be too small to activate glycolysis, whereby another mechanism...
(cdB3-Hb reaction and oxygenation coupling of the activity of glycolytic enzymes) may be needed.

Given the low ratio of band 3 proteins to tetrameric Hb molecules in red cells (about 1:50 in humans) (19, 37), the allosteric peptide-Hb interaction cannot significantly influence O₂ unloading from Hb in vivo. However, the small fraction of Hb molecules that could be affected is strategically positioned near the surface of the red cell membrane, where much of the chemistry affecting Hb-O₂ affinity appears to take place. Other evidence points to functional differentiation of the Hb molecules near the red cell membrane. The other (COOH terminal) cytoplasmic tail of band 3 binds carbonic anhydrase, which catalyzes the formation of HCO₃⁻ from CO₂ entering the red cells in tissue capillaries, and the local acidification caused by the liberated protons will initiate the release of O₂ by the Bohr effect in the immediate vicinity of band 3 (3). Similarly, the HCO₃⁻ generated by dissociation of carbonic acid is known to exchange via band 3 with extracellular Cl⁻, thereby increasing the local concentration of this modulator also. Furthermore, for the Hb molecules at the inner surface of the red cell membrane, the reduced cooperativity in the presence of subequivalent amounts of human band 3 peptide is interpreted (55) to increase O₂ saturation at low P O₂ (below P₅₀).

However, specific membrane characteristics that caution against freely extrapolating the in vitro observations to the intact red cells follow from observations that Hb may bind to band 3 as well as to non-band 3 sites (with high and low affinity, respectively), that band 3 occurs as dimers or tetramers in the red cell membranes, each dimer binding two Hb tetramers in an anticooperative fashion, and that the affinity constant for binding the second Hb molecule is almost 4 times lower than that for the first (8).

Other oxygenation-linked processes. Because the electro-neutral anion exchanger band 3 constitutes the membrane’s major organizing center, the oxygenation-dependent reaction with Hb may influence a range of other membrane properties and functions, although definitive evidence for Hb’s role as an O₂ sensor is still lacking (11). Despite a flexible link between the cytoplasmic and membrane domains of band 3 (43), the effects of Hb binding may extend far beyond the NH₂-terminal binding site, as is indicated by fluorescence quenching seen at the Lys(430) residue when Hb binds to “inside-out” vesicles from eosin-5-maleimide-labeled red cells (8). Also, adrenergic Na⁺/H⁺ exchange is more pronounced in deoxygenated than in oxygenated trout erythrocytes (29) and is inhibited when Hb is oxidized (33). Potassium fluxes also correlate with Hb oxygenation, and K⁺-Cl⁻ cotransport out of carp red cells is inactivated by deoxygenation but activated by oxygenation and stimulated by nitrate-induced formation of metHb, which has an R-like conformation (17, 18, 20). However, although band 3-mediated sulfate transport in human erythrocytes is twofold higher at high than at low Po2 and decreased by Mg at high and at low Po2 (10), the rate constants for unidirectional ⁸⁶Cl⁻ flux are unchanged in oxygenated and deoxygenated erythrocytes of the carp, trout, eel, cod, and human (19). Evidence for the oxygenation dependence of cellular rheology derives from the observation that adrenergic erythrocyte swelling modulates the viscosity of deoxygenated but not oxygenated trout blood (39).

Our data indicating negligible in vivo cdB3-Hb interaction in fish and pronounced interaction in mammals is in accordance with data on the O₂ dependence of red cell K⁺ fluxes.

Whereas the O₂ activation curve of K⁺-Cl⁻ cotransport in horse red cells suggests a role of membrane-bound Hb as O₂ sensor in mammalian red cells (9, 40), the data of Berenbrink et al. (1) on the O₂ sensitivity, cooperativity, and pH dependence of K⁺ fluxes provide strong evidence that bulk Hb is not the O₂ sensor in trout red cells.

The recent discovery that the cytoplasmic domain of band 3 drastically decreases the O₂ affinity of S-nitrosated Hb suggests that an intracellular S-nitroso-Hb-band 3 complex may play a role in targeting NO and that it may be linked to Heinz body formation (2). No direct information appears to be available on the possible effects of Hb oxygenation on band 3’s role in age-dependent clearance of red blood cells (36).

In conclusion, as illustrated in Fig. 12, the interaction of cdB3 with deoxyHb (Fig. 12, a) is ionic and similar to that of the endogenous erythrocyte phosphate effectors (Fig. 12, b), whereby cdB3 and the phosphates compete for deoxyHb in all vertebrate classes investigated. As with the Hb-phosphate interaction, the Hb-cdB3 interaction is inhibited by Mg²⁺ (Fig. 12, c and d, respectively). The docking of cdB3 peptides and of ATP/DPG at the same site of deoxyHb (in the entrance of the central cavity) implies some degree of stereochemical homology between cdB3 and the organic phosphates, which suggests that both these ligands may bind to a similar site on glycolytic enzymes (Fig. 12, e and f) and that these reactions might also be inhibited by divalent cations (Fig. 12, c and d). Apart from exerting its well-known modulatory effect on O₂ affinity, DPG inhibits ankyrin binding to band 3 (Fig. 12g) and reduces the number and affinity of protein 4.1 binding sites (28) and after its release from Hb on oxygenation may also modulate the mechanical properties of the erythrocyte membrane.

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