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Fluorescence Assay of the Interaction between Hemoglobin and the Cytoplasmic Domain of Erythrocyte Membrane Band3

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Abstract

Oxygen tension has emerged as a potent regulator of multiple erythrocyte properties, including glucose metabolism, cell volume, ATP release, and cytoskeletal organization. Because hemoglobin (Hb)[1] binds to the cytoplasmic domain of band 3 (cdb3) in an oxygen dependent manner, with deoxyHb exhibiting significantly greater affinity for cdb3 than oxyHb, the deoxyHb-cdb3 interaction has been hypothesized to constitute the molecular switch for all O_2-controlled erythrocyte processes. In this study, we describe a rapid and accurate method for quantitating the interaction of deoxyHb binding to cdb3. For this purpose, enhanced green fluorescent protein (eGFP) is fused to the COOH-terminus of cdb3, and the binding of Hb to the NH_2-terminus of cdb3-eGFP is quantitated by Hb-mediated quenching of cdb3-eGFP fluorescence. As expected, the intensity of cdb3-eGFP fluorescence decreases only slightly following addition of oxyHb. However, upon deoxygenation of the same Hb-cdb3 solution, the fluorescence decreases dramatically (i.e. confirming that deoxyHb exhibits much greater affinity for cdb3 than oxyHb). Using this fluorescence quenching method, we not only confirm previously established characteristics of the Hb-cdb3 interaction, but also establish an assay that can be exploited to screen for inhibitors of the sickle Hb-cdb3 interaction that accelerates sickle Hb polymerization.

Keywords

red blood cells; protein structure; hemoglobin; anion exchanger 1; FRET analysis

Introduction

Molecular oxygen is well known to modulate the biological properties of mature human erythrocytes. First, the activities of several ion transport pathways, including Na/K/2Cl cotransport, Na/H antiport, and KCl cotransport are regulated by O_2 pressure, the latter changing 20-fold between oxygenated and deoxygenated red blood cells (RBCs)[1–4]. Not surprisingly, this rapidly reversible O_2 modulation leads to O_2-dependent changes in cell

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hydration and volume[5,6], raising the question regarding how a diatomic molecule like O_2 can simultaneously control so many transporters? Second, erythrocyte deoxygenation stimulates ATP release from RBCs[7], leading to activation of purinergic receptors on endothelial cells, the consequent production of NO, and an ensuing vasodilation[8,9]. Deoxygenation also reversibly shifts glucose consumption from the pentose phosphate pathway (PPP) to glycolysis[10–12], acting as a switch to assure abundant reducing power (i.e. NADPH and glutathione) when the erythrocyte is saturated with O_2 and abundant ATP when it is not. Finally, RBC deoxygenation reversibly dissociates the major bridge connecting the membrane to its cytoskeleton by severing the linkage between ankyrin and band 3 (also known as AE1, the anion transporter, the most abundant protein in the RBC membrane), the major anchor of the cytoskeleton to the bilayer[13–16]. (Fig. 1).

Consideration of possible mechanisms to explain the above O_2-regulated processes prompted us to hypothesize that the reversible association of deoxyhemoglobin (deoxyHb) with band 3 might constitute a molecular switch that could trigger each of the above physiological changes. Indeed, there is considerable *in vitro* support for this hypothesis. First, deoxy- but not oxyHb has been found to bind avidly to the cytoplasmic domain of human band 3 (cdb3), specifically to residues 12–23 of the polypeptide[17,18]. Since no other O_2 binding protein has been identified in RBCs, and because the only established deoxyHb binding site in the membrane exists on band 3, the reversible association of deoxyHb with band 3 seemed like a plausible molecular switch. Second, a crystal structure of a complex between deoxyHb and the NH_2-terminus of cdb3 reveals that the NH_2-terminus of band 3 extends 18Å into a central cavity of deoxyHb[18]; i.e. the same cavity that closes upon Hb oxygenation. Thus, a mechanism immediately exists to explain why the deoxyHb-band 3 interaction is O_2 dependent. Multiple lines of evidence also suggest that this reversible association of deoxyHb with band 3 changes the global conformation of the anion transporter[19–21], displacing several signaling enzymes from cdb3[22–24] and thereby enabling communication of the oxygenation state of the cell to other membrane proteins. Moreover, band 3 has been shown to directly bind proteins thought to be involved in O_2-regulated processes, including syk and lyn tyrosine kinases[24–28], casein kinase I[29], tyrosine phosphatases[25,30], a glycolytic enzyme complex[31–33], ankyrin[34,35], protein 4.1[36,37], protein 4.2[38–40], adducin[41], two glycophorins[42,43], and several transporters[16,44]. In brief, the raw material for band 3 to mediate O_2 regulation of RBC functions is present in the human erythrocyte. However, to test whether band 3 was intrinsically involved, we required an assay that would allow us to identify mutations in band 3 that might eliminate band 3's affinity for deoxyHb and thereby the O_2 regulation of RBC function. The focus of this paper was therefore to design and optimize an assay for measuring the affinity of deoxyHb for band 3 in order to study the interaction under different conditions.

Materials and Methods

Materials

Dialysis was performed using dialysis bags from Spectrum. When desired, proteins were concentrated by ultrafiltration in Vivaspin tubes obtained from GE Healthcare Life Sciences, and final protein concentrations were assayed using a MicroBCA kit from Thermo Scientific

according to the manufacturer's instructions. This MicroBCA assay is based on reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline solution. Protease inhibitors were purchased from Research Products International. All other materials and reagents were purchased from Sigma-Aldrich.

Design of cdb3 – eGFP fusion proteins

In order to assay the interaction of Hb with cdb3, we required fusion proteins comprised of wild type and mutant cdb3s linked at their COOH-termini to enhanced green fluorescent protein (eGFP). Moreover, to facilitate purification of the expressed fusion proteins, a histidine (His_8) tag was attached to the COOH-terminus of each eGFP. The required cDNAs for murine cytoplasmic domain of band3 (corresponding to amino acids 1 to 398), murine kidney cdb3 (amino acids 80 to 398), and human cdb3 (amino acids 1 to 379) were PCR-amplified from the corresponding full length band 3 cDNA clones using forward primers containing an NdeI cleavage site followed by the start codon and reverse primers containing a XhoI cleavage site. These primers were:

1. for wild type mouse cdb3, forward: 5′-CATATGGGGGACATGCGGGACCAC-3′; reverse: 5′-CTCGAGAAAGATCCGGCCTGTGCG-3′
2. for mouse kidney cdb3, forward: 5′-GCGCATATGGACCAGAGGAACCAG-3′; reverse: 5′-CTCGAGAAAGATCCGGCCTGTGCG-3′
3. for human cdb3, forward: 5′-CGCCATATGGAGGAGCTGCAGGATGAT-3′; reverse: 5′-CTCGAGGAAGAGCTGGCCTGTCTG-3′.

Because the original eGFP gene contained an additional NdeI cleavage site within its coding sequence, we mutated this site to an alternate codon encoding the same amino acid. The amplified cDNA product was ligated into a pGEM-T easy vector (Promega), and after amplification, removed and inserted upstream of the eGFP sequence in a eGFP-fusion vector. Finally, all PCR-amplified cDNA fragments were sequenced to ensure fidelity of amplification.

Expression and purification of cdb3- eGFP proteins

Fusion protein expression was explored in various E. coli BL21(DE3) strains (Invitrogen) under the conditions described in the Results section. Optimal induction of fusion protein was obtained in BL21(DE3)pLysS bacteria grown in 2XYT media (16g tryptone, 10g yeast extract, and 5g NaCl per litter) at 28°C for 2 to 3 hours. The bacterial pellet was stored overnight at −80°C, thawed in lysis buffer (20mM NaH_2PO_4, 500mM NaCl, 15mM imidazole, 1 uM AEBSF, pH 7.5), and lysed on ice in a French press (SLM-Aminco). The bacterial lysate was centrifugated at 17,000×g in a Sorval SS-35 rotor for 30 minutes, and the supernatant was filtered and loaded onto a nickel-affinity column (GE Healthcare Life Sciences) equilibrated in lysis buffer. After loading, the column was washed with lysis buffer containing 40mM imidazole and then eluted in a gradient of the same buffer increasing to 250mM imidazole.

Hemoglobin preparation and handling

All animal procedures were approved by Purdue Animal Care and Use Committee in accordance with the guidelines from the National Institute of Health. Human blood was obtained with the understanding and written consent of each subject. The study methodologies were approved by the Purdue Institutional Review Board and conformed to the standards set by the Declaration of Helsinki. Human and murine erythrocyte hemoglobin were obtained from fresh whole blood as described previously[11]. Briefly, plasma and the buffy-coat were removed by centrifugation, and RBCs were washed in PBS (137mM NaCl, 2.7mM KCl, 8.1mM NaH_2PO_4, 1.5mM KH_2PO_4, pH 7.4). The packed RBCs were stored at 4°C for one week to begin depletion of 2,3-DPG, after which the cells were lysed in 5 volumes of cold water and spun at 20,000×g at 4°C. To remove any residual 2,3-DPG, the supernatant was first dialyzed for 12h in 1M NaCl, then 0.1M NaCl, and finally in 10 mM BisTris acetate, pH 6.5. Hemoglobin concentration was determined by measuring absorbance at 540nm using an extinction coefficient of 14.17 liters $mmol^{-1}$ cm^{-1} [45]. Deoxygenation was performed by flushing the Hb solutions in a sealed cuvette with humidified argon until the change in absorption spectrum confirmed maximal deoxygenation of the sample (Fig. 2 insert, full absorption spectrum is not shown)[45]. Purification of hemoglobin to homogeneity was not routinely done, since initial studies demonstrated that deoxyHb binding to cdb3 is not affected by further purification steps.

Fluorescence resonance energy transfer (FRET) analysis of Hb affinity for band 3

All fluorescence measurements were obtained on an AMINCO-Bowman luminescence spectrometer, using an excitation wavelength of 380 nm and an emission spectrum scanned from 475 to 550 nm. The emission spectrum of the fully oxygenated solution of cdb3-eGFP plus hemoglobin was recorded first, and then the same solution was deoxygenated and the emission spectrum recorded again. The control sample with free eGFP (not fused to cdb3) was used to assess the magnitude of inner filter effects, as discussed under the Results section.

Calculation of the Forster distance between Hb and eGFP

R_0, the Forster distance, is defined as the distance at which energy transfer efficiency from a fluorescence donor to a fluorescence acceptor pair is 50%. This R_0 value depends both on the overlap integral of the emission spectrum of the donor with the absorption spectrum of the acceptor and their mutual molecular orientations. For the eGFP-Hb pair, R_0 was computed to be 5 nm, according to the formula

$$R_0 = 0.211 \left[\frac{k^2 Q Y_D J}{n^4} \right]^{1/6}$$

where k is the orientation factor between donor and acceptor (in general, it is assumed that $k^2 = 2/3$), n is the refractive index (1.33 for water, 1.4 for proteins), QY_D is the fluorescence quantum yield of the donor in the absence of the acceptor (0.79 for eGFP) and J is the overlap integral. J was calculated using the a|e - UV-Vis Spectral Software 1.0 (www.FluorTools.com). The values for k^2, QY_D and n were obtained from[46].

Results

Design of a band 3 reporter molecule that changes fluorescence upon binding of deoxyHb

In order to determine the impact of band 3 mutations on the relative affinity of band 3 for oxy- versus deoxyHb, we needed an assay that would generate a strong signal upon hemoglobin binding. For this purpose, we fused enhanced green fluorescent protein (eGFP) to the COOH-terminus of the cytoplasmic domain of band 3 (cdb3) with the expectation that binding of Hb to the NH_2-terminus of cdb3 would lead to quenching of eGFP fluorescence at the NH_2-terminus. Our reason for anticipating a change in eGFP quantum yield upon Hb binding was that the deoxyHb binding site in the crystal structure of human cdb3 resides only 1.8nm from the site of eGFP fusion to cdb3. Because the R_0 for the eGFP-Hb FRET couple is only 5 nm, one would expect eGFP quenching upon hemoglobin binding.

Although the fluorescence quencher had to be Hb, we selected eGFP as the fluorescence donor for two important reasons. First, the emission spectrum of eGFP overlaps significantly with the absorption spectrum of both oxyHb and deoxyHb (Fig. 2). Second, we needed a fluorescence donor with both an emission and excitation maximum at wavelengths where oxy- and deoxyHb exhibit identical absorbances (i.e. to avoid any differences in the absorption of the fluorescence excitation and emission of eGFP during changes in Hb oxygenation). Comparison of the absorption spectra of oxy and deoxyHb revealed that at most wavelengths their absorption spectra are different (Fig. 2). However, at 380 nm and 510 nm eGFP can be excited and emit fluorescence without any differential absorption by oxy- or deoxyHb, respectively. Thus, by setting the excitation and emission monochrometers to 380 and 510 nm, respectively, changes in Hb oxygenation will not affect the FRET analyses unless the changes in oxygenation alter Hb binding and thereby affect eGFP quenching.

Expression, purification and characterization of cdb3-eGFP fusion proteins

The expression vector for the cdb3-eGFP fusion protein was prepared from a commercial vector containing a T7 polymerase promoter upstream from a multi-cloning site linked to the eGFP gene. As explained in the Methods, the cdb3 gene was inserted into this multi-cloning site between NdeI and XhoI restriction sites, resulting in the desired fusion protein with eGFP attached to residue 379 or 398 of the COOH-terminus of human or mouse cdb3, respectively. To assure optimal protein expression, the vector was transformed into E. coli strain BL21(DE3) containing the T7 polymerase gene and an IPTG inducible promoter. Initial attempts to express cdb3-eGFP (37°C for 5 hours) were unsuccessful due to its rapid proteolysis. To remedy this problem, we elected to: i) prevent protein expression in the absence of IPTG by using an E. coli strain containing T7 lysozyme (pLysS strain), which is an inhibitor of T7 polymerase; ii) reduce fusion protein digestion during IPTG induction by stimulation with IPTG at a reduced temperature (28°C), iii) inhibit further cdb3-eGFP proteolysis by inclusion of 1mM AEBSF in the lysis buffer; and iv) shorten the length of the IPTG induction time from 5 to 3 hours. With these modifications, acceptable levels of both human and murine cdb3-eGFP fusion proteins could be expressed and purified (Fig. 3).

Use of FRET quenching to evaluate hemoglobin binding to cdb3-eGFP

Because of the intense absorption of Hb, Hb binding to cdb3-eGFP had to be performed in very dilute solutions (Hb=0.07–8μM; [cdb3-eGFP]=0.2–0.5 μM). However, to assure that binding would still occur at these low protein concentrations, solution conditions were selected to maximize Hb-cdb3 affinity. Based on previously published observations[47,48], these conditions required use of low ionic strength and slightly acidic pH. Thus, for all FRET analyses, both proteins were dialyzed overnight against 10 mM BisTris-acetate buffer, pH6.5.

As seen in Fig. 4, both eGFP alone and cdb3-eGFP yielded the maximum relative fluorescence in the absence of Hb. Addition of oxyHb to cdb3-eGFP resulted in significant (20%) eGFP quenching, probably due to a combination of some FRET-mediated quenching due to weak oxyHb binding and inner filter effects from free Hb. The latter effect commonly arises when molecules present throughout the solution absorbs either excitation or emission light[49]. Comparison of the absorption spectrum of Hb with the excitation and emission spectra of eGFP confirms that Hb will absorb both excitation and emission light (Fig. 2).

To determine the fraction of fluorescence quenching due to inner filter effects, NaCl was added to induce dissociation of the Hb-cdb3 interaction[47,48], thereby removing any contribution of FRET-induced quenching from the total decrease in eGFP fluorescence. As seen in Fig. 4, addition of NaCl to the cdb3-eGFP and Hb mixture returned cdb3-eGFP fluorescence to nearly its nonquenched control value, suggesting that most of the oxyHb-induced quenching of cdb3-eGFP is due to direct binding of the two proteins. To confirm that only a small fraction of the Hb-induced quenching of cdb3-eGFP is due to inner filter effects of Hb, a similar set of measurements were obtained on free eGFP solutions in the absence of cdb3. As seen in Fig. 4, eGFP alone displayed the same fluorescence as cdb3-eGFP alone, but addition of Hb lowered the fluorescence of the eGFP solution only to the level of the Hb and cdb3-eGFP solution in the presence of NaCl. We therefore conclude that inner filter effects contribute only a small fraction of the total quenching seen upon addition of Hb. Nevertheless, to accurately correct for any inner filter effects in our calculations of FRET quenching due to Hb binding, the following formula was used:

$$\%\mathrm{Hb\,quenching} = \left\{1 - \left(\mathrm{F}_{\mathrm{cdb3-eGFP+Hb}}/\mathrm{F}_{\mathrm{eGFP+\ Hb}}\right)\right\} * 100$$

where F is the fluorescence of cdb3-eGFP ($F_{protein}$) or free eGFP (F_{gfp}) in the presence of oxy or deoxyHb.

Analysis of the binding affinity of Hb for cdb3-eGFP was performed by measuring the quenching of cdb3-eGFP as a function of Hb concentration and plotting the data using a standard noncooperative single binding site algorithm to determine K_d. Evaluation of the binding of oxyHb to cdb3-eGFP under the low ionic strength conditions described above (Fig. 5) reveal K_d values for human and mouse cdb3 of 1.8×10^{-7} M and 5.5×10^{-7}M, respectively. These results are similar to those obtained under related low ionic strength conditions for binding of oxyHb to isolated erythrocyte membranes[50].

Analysis of the effect of oxygen on Hb binding to cdb3-eGFP was performed with the same cdb3-eGFP and Hb mixtures, only the solutions were subsequently deoxygenated by exposure to a stream of humidified argon for 45 min. In this protocol, the fluorescence intensity of the oxygenated sample was recorded first and then the same sample was deoxygenated and the fluorescence intensity recorded again. Deoxygenation was then confirmed by determining the absorption spectrum of the sample. As shown in Fig. 6, quenching of the cdb3-eGFP and deoxyHb sample was significantly greater than quenching of the corresponding oxyHb sample, suggesting that differential quenching can be used to map the binding site of deoxyHb on cdb3. To confirm this contention, cdb3-eGFP lacking residues 1–79 of murine cdb3 was examined and found to exhibit little deoxygenation-induced fluorescence quenching (fig. 6). Because deletion of residues 1–79 removes residues that contain the previously identified deoxyHb binding site on murine cdb3, the absence of deoxyHb binding to this deletion mutant confirms the validity of the analytical procedure.

Discussion

In this study, we have developed a FRET assay to quantitate the interaction of Hb with cdb3. For this purpose, eGFP was fused to the COOH-terminus of cdb3 at a distance of 1.75 nm from the docking site for Hb at the NH_2-terminus, based on crystal structure calculations[51]. Since this distance between sites is $<R_0$ (i.e. 5 nm), the interaction between Hb and cdb3 can be studied by measuring FRET-mediated quenching of eGFP. We believe this FRET assay may be preferred over other methods for quantitating the O_2-dependence of Hb-cdb3 interactions[47,48] for the following reasons: 1) in comparison to previous pull-down assays, our FRET assay is not encumbered by non-specific interactions between Hb and the substrate (e.g. beads) used to immobilize cdb3; 2) omission of all washing steps in our FRET assay avoids loss of weakly bound Hb which must be measured when investigating binding to low affinity cdb3 mutants; 3) very low concentrations of protein are required for this assay, rendering it especially suitable for valuable or difficult to obtain mutant proteins; 4) because the assay is performed in a sealed cuvette that can be repeatedly oxygenated and deoxygenated through inserted ports, the reversibility of the interaction can be accurately evaluated; and 5) using a multi-well plate and a plate reader with a gas-controlled detection chamber (e.g. infinite 200 PRO plate reader from Tecan), the assay may be adaptable to high throughput screening applications.

One possible use of the potential drug screening application of this assay arises from the observation that cdb3 accelerates the polymerization of deoxygenated sickle hemoglobin (deoxyHbS) in sickle cell disease[52]. This rate of HbS sickling is critical to manifestation of disease symptoms, since if sickling can be postponed until the erythrocyte reaches venous circulation, where the cells are not constrained by a narrow vasculature, the cells can reach the lungs where reoxygenation can induce HbS depolymerization. In order to identify drugs that might prevent HbS sickling and thereby treat sickle cell disease, an assay of small membrane-permeable molecules for their abilities to block deoxyHb-cdb3 interactions might prove useful.

Finally, because the NH_2-terminus of cdb3 is known to be the anchor of numerous erythrocyte membrane proteins, a slightly modified version of our FRET assay could prove

useful for characterization of a number of cdb3-peripheral protein interactions. For example, the association of glycolytic enzymes[32], protein 4.1[36], protein 4.2[38], ankyrin[34], and syk and lyn tyrosine kinases[28] with cdb3 should all be measurable using this method as long as the aforementioned protein can be labeled with an appropriate FRET pair for cdb3-eGFP. By this method, many otherwise difficult to study cdb3 interactions can now be quantitatively evaluated.

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Abbreviations

PPP pentose phosphate pathway

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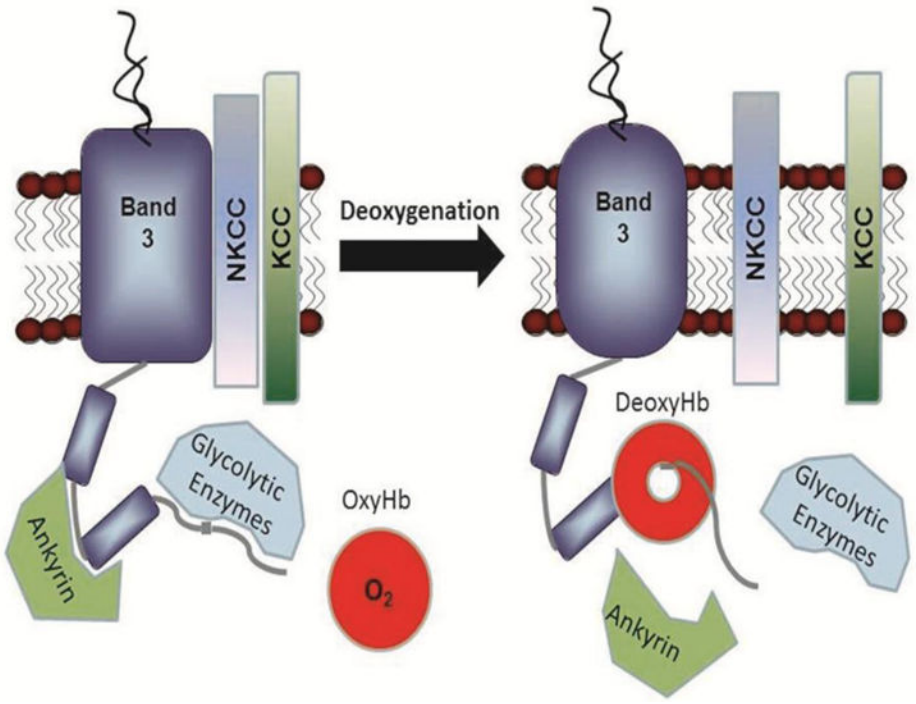

Fig. 1. Oxygen regulation of erythrocyte membrane protein interactions

Glucose metabolism[11], cation transport[1], ATP release[7] into circulation and ankyrin binding[34] have all been reported to be oxygen-dependent processes in erythrocytes. Because the cytoplasmic domain of band 3 (cdb3) constitutes the only known Hb binding site on the erythrocyte membrane and since the Hb-cdb3 interaction is strongly O_2-dependent, many if not all oxygen-regulated pathways in erythrocytes have been postulated to depend on the reversible association of deoxyHb with cdb3[17]. This sketch depicts the binding of deoxyHb to cdb3 and the consequent displacement of several cdb3-associated proteins. Published crystal structure data reveal that deoxyHb binds cdb3 like a “donut on a string”, with the NH_2-terminus of cdb3 extending 1.8 nm into the central cavity of deoxyHb[51].

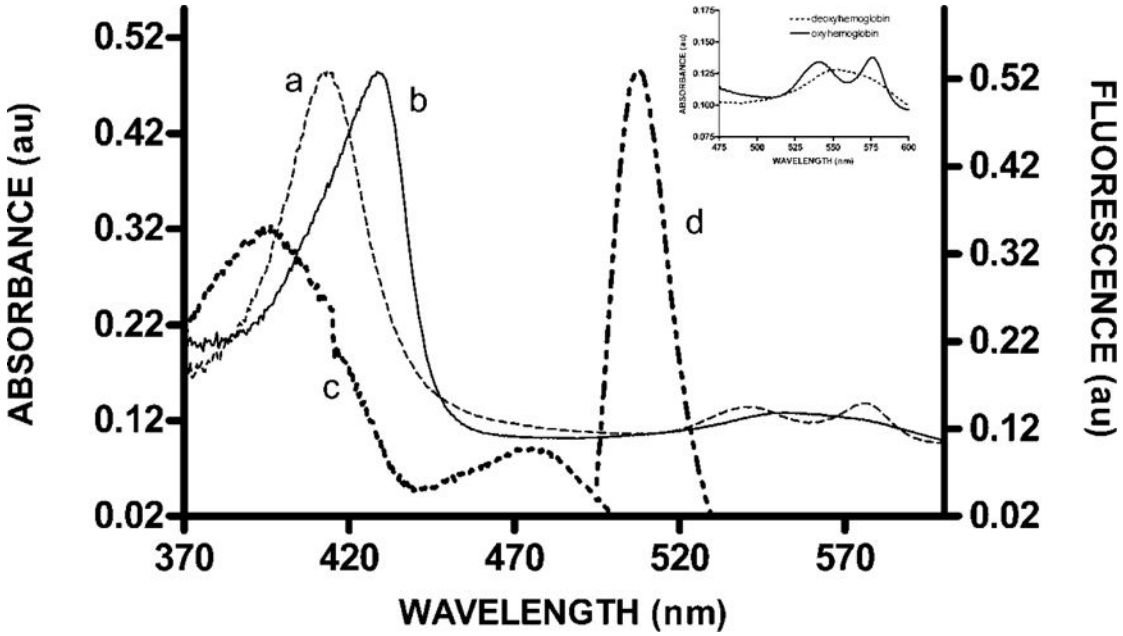

Fig. 2. Selection of a fluorescence resonance energy transfer donor for FRET quenching by Hb using spectral analysis of oxy- and deoxyHb

The absorption spectra of oxyHb (a) and deoxyHb (b) are identical at 380nm and 510nm. To prevent differential absorption of either donor excitation or emission as a function of oxygen tension, a FRET donor was required that could be excited at 380nm and would then emit at 510nm. As demonstrated by the excitation (c) and emission (d) spectra of eGFP, this protein complies with these spectral requirements and was therefore selected as the FRET donor. To show complete deoxygenation under our conditions the absorbance spectra of hemoglobin between 475–600nm before and after deoxygenation are shown as an inset in this figure.

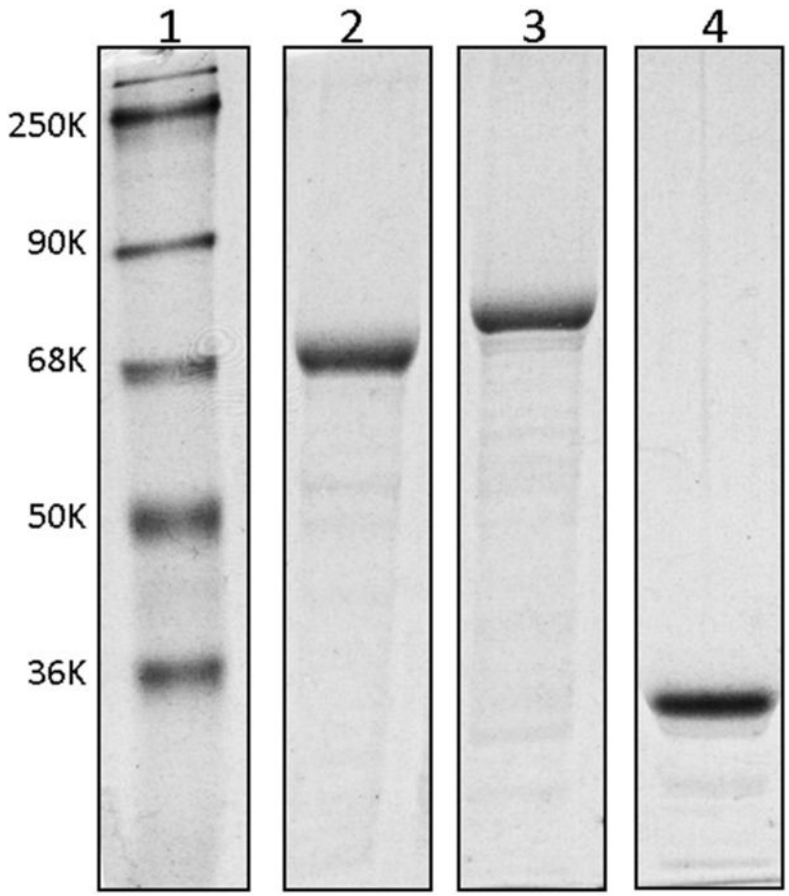

Fig. 3. SDS-PAGE analysis of various cdb3-eGFP constructs following expression and purification from bacteria

Cdb3-eGFP proteins were expressed in E. coli strain BL21(DE3) pLys S, purified using an affinity column, and analyzed by SDS-PAGE: lane 1, molecular markers; lane 2, human cdb3-eGFP fusion protein containing human cdb3 residues 1–379; lane 3, mouse cdb3-eGFP fusion protein containing mouse cdb3 residues 1–398; lane 4, eGFP protein which is not fused with cdb3.

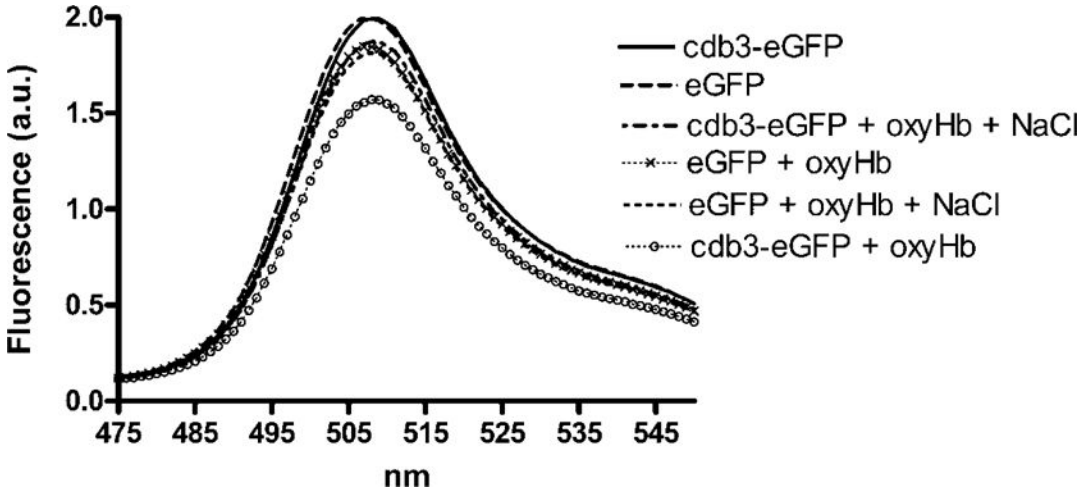

Fig.4. Quenching of cdb3-eGFP fluorescence by human oxyHb under various conditions

Either cdb3-eGFP or eGFP alone were incubated in 10 mM BisTris buffer (pH 6.5) under the following conditions: 1) in the absence of Hb; 2) in the presence of Hb; 3) in the presence of Hb and 50 mM NaCl. The emission spectrum was then recorded from 475nm to 550nm following excitation at 380nm. Each experiment was analyzed in triplicate with essentially identical results.

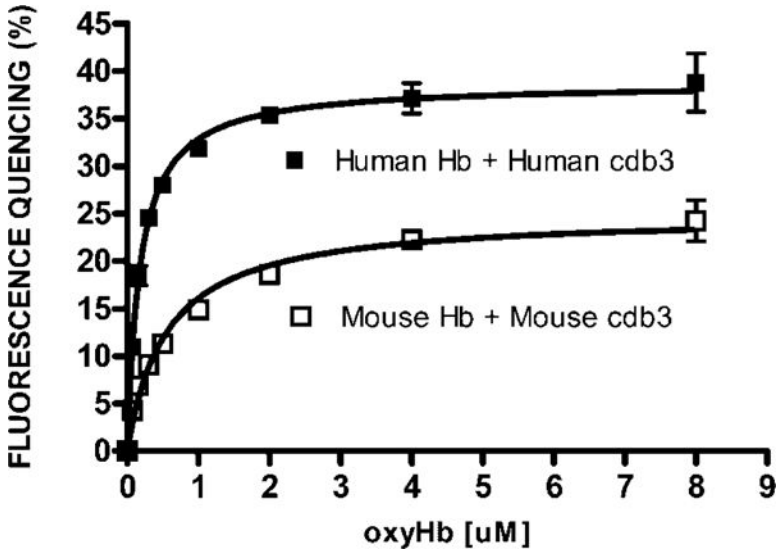

Fig. 5. Measurement of Hb binding to cdb3-eGFP by analysis of FRET

Increasing concentrations of Hb (0.07μM to 8μM) were incubated for 30 min at room temperature with cdb3-eGFP (0.2μM) in 10 mM BisTris buffer (pH6.5). Emission spectra were then recorded and the fluorescence intensity at 510nm was used to calculate the % fluorescence quenching using the equation described in the text. Prism software was employed to determine a K_d of human oxyhemoglobin for human cdb3 of 1.8×10^{-7} M; and the K_d of murine oxyhemoglobin for murine cdb3 of 5.5×10^{-7}M. The data constitute the mean +/− S.D. of two experiments.

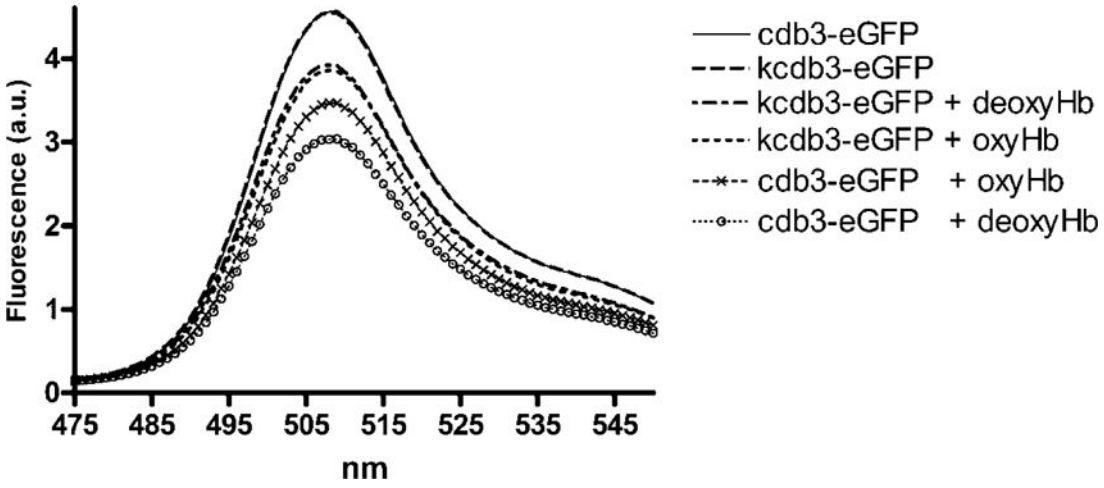

Fig. 6. Measurement of the effect of oxygen on murine cdb3-eGFP binding to murine hemoglobin by FRET

0.5μM of cdb3 was incubated with 1μM Hb under atmospheric conditions, and the emission spectrum of the solution was recorded. This same solution was then deoxygenated under a stream of humidified argon, and the emission spectrum of the solution was again recorded. Deoxygenation was confirmed by monitoring the absorbance of the solution. The kidney spliceoform of cdb3 (kcdb3) was used as a negative control, because it lacks the entire NH_2-terminus of cdb3 (residues 1–79) required for deoxyHb binding.