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Fluorescence assay of the interaction between hemoglobin and the cytoplasmic domain of erythrocyte membrane band 3

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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.

1. 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 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 18 Å 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

Abbreviations: PPP, pentose phosphate pathway.

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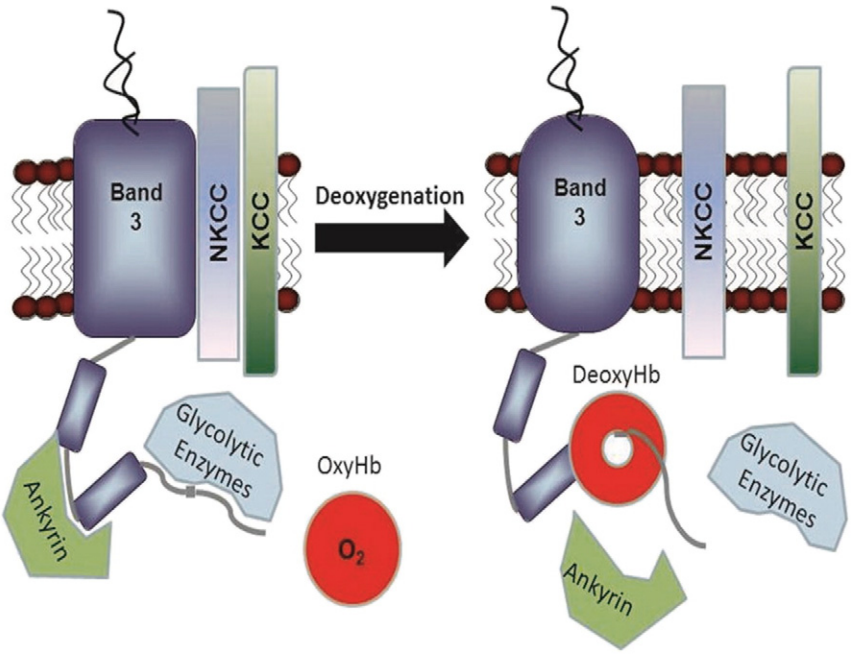

Fig. 1. *Oxygen regulation of erythrocyte membrane protein interactions.* Glucose metabolism [11], cation transport [1], ATP release [7] into circulation and ankyrin binding [34] have all been reported to be oxygen-dependent processes in erythrocytes. Because the cytoplasmic domain of band 3 (cdb3) constitutes 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].

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.

2. Materials and methods

2.1. 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.

2.2. 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 band 3 (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′; and
3.) for human cdb3, forward: 5′-CGCCATATGGAGGAGCTGCAGGATGAT-3′; reverse: 5′-CTCGAGG AAGAGCTGGCCTGTCTG-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.

2.3. Expression and purification of cdb3- eGFP proteins

Fusion protein expression was explored in various *Escherichia 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 (16 g tryptone, 10 g yeast extract, and 5 g NaCl per litter) at 28 °C for 2 to 3 h. The bacterial pellet was stored overnight at −80 °C, thawed in lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, 15 mM 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 min, 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 40 mM imidazole