Oxygen-linked modulation of erythrocyte metabolism: state of the art

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Introduction

About fifteen years ago a number of experimental data on the specific interaction of haemoglobin (Hb) and various enzymes of the glycolytic pathway with the cytoplasmatic domain of band 3 protein emerged[1-3]. These data induced our research group to formulate the hypothesis of a modulation of the erythrocyte (RBC) metabolism driven by the free energy connected to the R to T Hb transition[4]. The general scheme of this simple model is reported in figure 1. In this model band 3 protein plays a pivotal role: when the erythrocyte is at high oxygenation state (HOS) band 3 interacts with some glycolytic enzymes (inhibiting their activity) and more glucose is addressed towards the pentose phosphate pathway (PPP). When the erythrocyte is at low oxygenation state (LOS) the high affinity of deoxy-Hb for the N-terminal cytoplasmatic domain of band 3 induces the release of glycolytic enzymes, and thus glucose flux towards the glycolytic pathway increases. Some recent results obtained by our and other groups are in agreement with this simple model, even though they indicate, that other factors might play a significant role in this erythrocyte modulation. The aim of this brief review is to offer to the reader a critical examination of the significance of these recent results in light of the model framework.

Band 3 protein

Band 3 is a transmembrane protein that accounts for about 25% of the total RBC membrane proteins. It is characterized by three distinct functional domains:

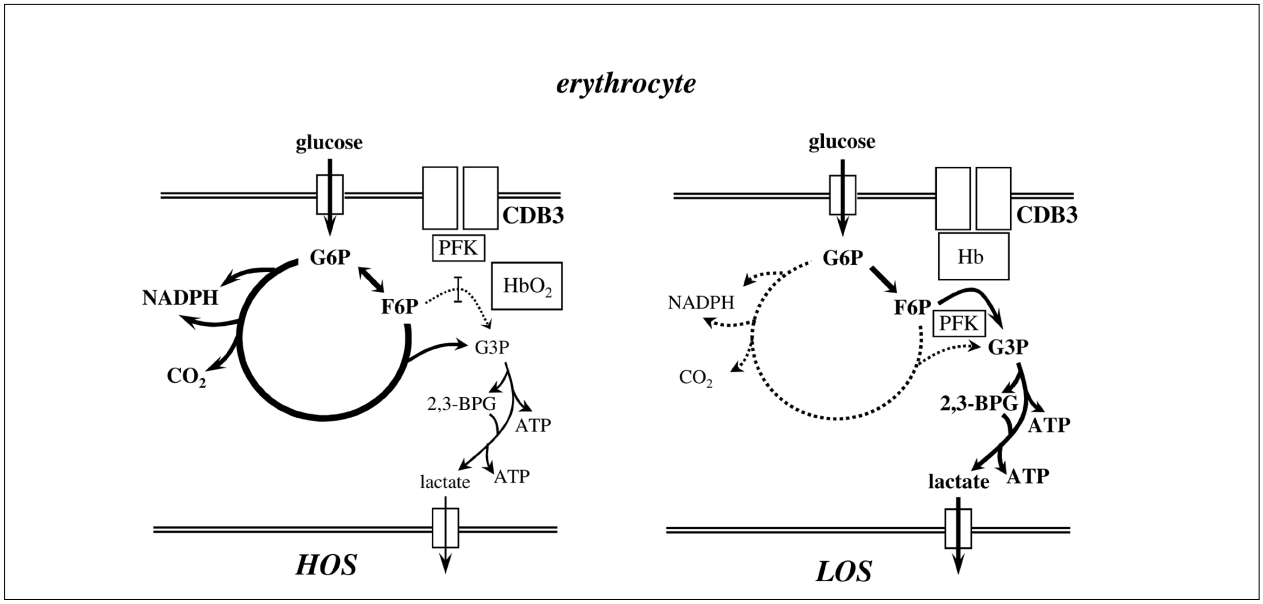

Figure 1 - Simplified scheme representing the modulation of erythrocyte metabolism by the O_2 transition of Hb and its competition with glycolytic enzymes (mainly phosphofructokinase, PFK) for the cytoplasmic domain of band 3 (CDB3).

Blood Transfus 2010; 8 Suppl 3:s53-s58 DOI 10.2450/2010.009S

(1) the membrane spanning domain, which transverses 12 times the bilayer and serves to catalyze the exchange of anions (mainly Cl^- and HCO_3^-) across the membrane[5]; (2) the short C-terminal cytoplasmatic domain that binds carbonic anhydrase II[6,7], and (3) the N-terminal cytoplasmatic domain (CDB3) that binds a variety of proteins[8] and anchors the RBC membrane to the underlying cytoskeleton via ankyrin and protein 4.2[9].

Glycolysis and pentose phosphate pathway flux measurements

Initially, to confirm the reported hypothesis, metabolic behavior of human RBC under conditions in which they are fully oxygenated (HOS conditions) or greatly deoxygenated (LOS conditions) has been investigated. The results evidenced that glucose consumption is strongly pH dependent, but almost independent upon the oxygenation state[10,11]. However, the ratio between $^{14}CO_2$ production and glucose consumption corresponding to the fraction of glucose utilized through the PPP resulted 2-fold higher in HOS than in LOS erythrocytes at any pH value, showing an increase for both oxygenation states as pH decreased. Moreover, measurement by ^{13}C-NMR spectroscopy of the rate of [^{13}C-3] lactate production in RBCs incubated with ^{13}C-1-labelled glucose that is linked only to glycolysis, resulted significantly higher in erythrocytes kept in LOS. On the whole, the data obtained evidenced that HOS and LOS erythrocytes displayed a significant difference in the amount of glucose addressed towards PPP and glycolysis and that PPP is about two times more active in HOS samples. This modulation did not alter total glucose consumption, which is only pH-dependent. The results obtained were in perfect agreement with the hypothesis that the glycolytic flux is modulated via a competition between glycolytic enzymes and deoxy-Hb for CDB3. In fact, in HOS erythrocytes the inhibition of glycolytic enzymes, due to their binding to CDB3, should result in a reduced glucose flux through glycolysis. As a consequence, since glucose consumption is constant, more glucose is metabolized by the PPP in order to ensure adequate levels of NADPH necessary to protect the RBCs from oxidative stress deriving from the high oxygen load. Conversely, in the LOS state the displacement of glycolytic enzymes from CDB3, induced by the binding of deoxy-Hb, results in an increased glucose flux throughout glycolysis, thereby increasing ATP and 2,3-BPG production.

To evaluate the effect of CDB3 tyrosine phosphorylation on glucose metabolism, metabolic pathway fluxes have been recently measured in intact RBCs after pervanadate treatment[12]. Pervanade inhibits phosphotyrosine phosphatase, responsible for the dephosphorylation of tyrosine residues, and increases the activity of two tyrosine kinases, the $p72^{syk}$ and a kinase belonging to the Src family (most probably Lyn)[13,14], resulting in increased tyrosine phosphorylation. Phosphorylation of the tyrosine residues of band 3 at position 8 and 21, both involved in the N-terminal binding site, caused an increase of the oxygenated RBC glycolytic flux (45%) and a reduction (66%) of PPP flux with respect to control flux[12]. Confocal microscopy experiments evidenced also that pervanadate treatment of RBC caused the displacement of glycolytic enzymes from the membrane[15]. However, considering that deoxy-Hb binding site for band 3 is involved also in 2,3-BPG binding[16], and that the increased number of negative charges on the N-terminal domain of band 3, due to its phosphorylation, may greatly enhance Hb binding to band 3, it is conceivable to hypothesize that several Hb molecules (located in proximity of the RBC membrane) may undertake a deoxygenation-linked binding to phosphorylated band 3, with the consequent displacement of the glycolytic enzymes from CDB3. Anyway, the possibility of proving by *in vivo* experiments that this is the case is really difficult. Moreover, as far as we are aware of, no papers reporting an increased affinity of Hb for the tyrosine-phosphorylated CDB3 or for any tyrosine-phosphorylated peptide mimicking the N-terminus of band 3 have been published up to now.

To obtain further evidences on the involvement of the Hb-CDB3 interaction in the oxygen-linked modulation of erythrocyte metabolism, rate of ^{13}C-3 lactate production in RBCs incubated with ^{13}C-1-labelled glucose was measured by ^{13}C-NMR spectroscopy on RBCs saturated with carbon monoxide (CO), and on RBCs incubated at low oxygen pressure and treated with 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS), a strong inhibitor of band 3 function[10]. In both cases the difference between the two oxygenation

states almost vanished. In conclusion, the experiments on DIDS-treated erythrocytes supported the involvement of band 3 in the metabolic differences observed between oxygenated and deoxygenated RBCs, while those obtained on CO-saturated erythrocytes strongly suggested that the interaction with CDB3 involved the T conformation of Hb. In the presence of CO the Hb molecule is indeed blocked in the fully liganded R conformation even at very low oxygen tension. However, it should be underlined that no CDB3 phosphorylation level measurement was carried out in these experiments.

Haemoglobin and the band 3 complex

The differential interaction between band 3, oxygenated and deoxygenated Hb was evidenced by an experiment performed in the presence of increasing concentrations of the acetylated peptide corresponding to the first 11 residues of human band 3, that showed that the oxygen affinity of human Hb is progressively decreased[16]. Furthermore, the X-ray crystallographic analysis of this complex indicated that the peptide did not simply lie across the 2,3-BPG binding site (as it has been speculated in previous studies) but it penetrated deeply into the deoxy-Hb tetramer extending through the 2,3-BPG binding site, even if only from 5 to 7 of its 11 residues appeared in direct contact with β subunits. The other residues that extended out of the central cavity appeared highly disordered and thus, not visible in the electronic density map[16].

To confirm that this interaction is present also in different vertebrates, a series of peptides corresponding to the 10 N-terminal CDB3 residues of trout, chicken and human have been tested in their interaction with *Haplosternum littorale* cathodic Hb, Andean frog Hb, avian Hbs A and D, and the results confirmed that this interaction is ubiquitous in vertebrates[17].

In order to determine the precise binding site to band 3 in deoxy-Hb, a series of site-directed mutants of band 3 have been expressed in *Escherichia coli* and the measured shift of the oxygen binding curve of Hb has been compared to that obtained in the presence of the entire recombinant band 3 ($\Delta\log P_{50}$ = 18 mmHg)[18]. Band 3-lacking the first 12-23 residues (or a higher number of residues) did not significantly perturb the O_2 dissociation curve with respect to the measurements carried out in the absence of band 3, suggesting that residues 12-23 are essential for deoxy-Hb binding[18]. In this respect, the effect exerted on the oxygen affinity of Hb by the peptide corresponding to the first 11 residues of band 3 can be explained by the high similarity of the sequences 1-11 and 12-23 of band 3. However, experiments performed by using wild type and mutant isoforms of CDB3 demonstrated that the presence of residues 1-11 failed to enhance CDB3 interaction with deoxy-Hb, suggesting that the first 11 residues somehow inhibit the access of deoxy-Hb to its higher affinity site on residues 12-23 of CDB3. Moreover, mutation of the acidic amino acid residues surrounding Tyr21 to their uncharged counterparts caused a significant reduction of band 3 affinity for deoxy-Hb, and the additional substitution Tyr21→Ala reduced the affinity even more. Moreover, deletion of sequences on both sides of residues 12-23 of band 3 functioned to suppress, rather than to enhance, Hb-band 3 affinity[18].

Band 3 interactions

Although band 3 binding sites for a number of proteins have been precisely described, the whole network of interactions has not been studied in detail yet[8,19]. However, different studies described the interaction of many glycolytic enzymes to CDB3. The major band 3 binding site for aldolase has been shown to be localized within the first N-terminal 23 residues[19,20]. Two binding sites of glyceraldehyde 3-phosphate dehydrogenase (G3PD) are located at residues 1-11 and 12-23. The phosphofructokinase (PFK) binding site involves residues 12-23[19], while a direct interaction between band 3 and either pyruvate kinase (PK) and lactate dehydrogenase (LDH) has not been documented up to now[19], even if evidences of LDH-interaction with band 3 were already shown by Harris and Winzor 20 years ago[21].

The association between membrane and cytoskeleton is obtained through the interaction of band 3 (in tetrameric form) with ankyrin at the β-hairpin loop comprising residues 175-185[22], and also through the interaction of CDB3 (in tetrameric form) with protein 4.1. This interaction is alternative to that of protein 4.1 with glycophorins, that requires the presence of polyphosphoinositides as cofactors[23]. Another important site of interaction between the membrane and the spectrin-based cytoskeleton is

represented by the band 3 macrocomplex, consisting of Rh-associated glycoprotein, Rh polypeptides, CD47, LW, glycophorin B and Landsteiner-Wiener blood group protein[24].

The binding site of protein 4.2 on the N-terminus of CDB3 is still unknown, even if this protein has been proven to directly bind CDB3[25,26]. The binding site of glycophorin A for band 3 has been hypothesized to be located in the vicinity of the trans-membrane span 8 and 9 and also of the cytosolic C-terminus[27]. Carbonic anhydrase II, that catalyzes the hydratation of carbon dioxide to bicarbonate ions in RBC, is known to bind band 3 at the C-terminal domain, at the sequence L^{886}DADD[28]. Bicarbonate ions are channelled to band 3, which immediately exports it from the cell.

The influence of Hb-CDB3 interaction on the oxygen affinity of Hb may appear of reduced importance considering that only 0.5% of RBC's Hb molecules may bind band 3 in RBCs at LOS, due to the presence of 270 millions of Hb molecules and 1.2 million of band 3 molecules[29]. However, the impact of Hb-CDB3 interaction on nitric oxide (NO) delivery from RBC may be substantial[18]. In fact, it has been shown that the oxygen-linked intra-molecular transfer of some NO molecules bound to the heme Fe^{2+} to Cysβ93 (in form of an S-nitrosothiol) is followed by transfer of NO (transnitrosation) to CDB3 Cys residues, to which Hb is bound at LOS[30]. Thus, CDB3 may act as an allosteric regulator that promotes the T state of Hb and as an acceptor of NO groups transferred by SNO-Hb upon R to T transition. The number of band 3 molecules per RBC is 10 fold higher than that of SNO-Hb molecules in arterial RBC, suggesting that the transfer of NO to CDB3 may represent a main route in NO trafficking[30].

Moreover, considering the number of molecules of G3PD (500.000), aldolase (20.000) and PFK (6000) per RBC[31], it strongly emerges that the band 3 molecules are sufficient for binding all the molecules of the reported enzymes at HOS, and for displacing them during RBC deoxygenation, because of the competition with deoxy-Hb for CDB3.

Glycolytic Enzymes Localization *in vivo*

Confocal microscopy of unstimulated, healthy, intact and freshly drawn RBCs showed that the localization of PK and LDH, as well as aldolase, PFK and G3PD is at the level of the membrane[15], while after RBC deoxygenation, all these enzymes are displaced from the membrane to the cytoplasm, thus, confirming that the association of the enzyme complex to the membrane is regulated by the oxygenation state of RBC.

Furthermore, band 3 knock-out mice presented glycolytic enzymes localized throughout the cytoplasm and not membrane-bound, independently from the oxygenation state of the erythrocyte[32].

Metabolomics and transporter regulation

A dynamic mathematic model of RBC metabolism, involving the O_2 sensing mechanism of Hb and the hypoxia-induced activation of glycolytic enzymes correlated with their release from CDB3 was developed to predict temporal alterations in intracellular metabolites and cellular energetics in response to hypoxia[33]. It resulted that the alterations predicted by this updated version of the model are consistent with the metabolome analysis carried out by capillary electrophoresis mass spectrometry[34,35]. However, it is important to underline that a previous version of the model, that did not consider the effect of the Hb-CDB3 interaction, was unable to reproduce the experimental metabolic alterations observed[33].

The oxygenation state of RBCs resulted effective also in regulating the activity of many membrane soluble transporters, in fact the activity of the K/Cl cotransporter increased 20 times in oxygenated RBCs[36,37], while that of the Na-K-2Cl cotransporter and of the Na/H exchanger increased upon deoxygenation[37,38].

Conclusions

The fact that RBCs regulate their metabolism depending on their oxygenation state, favoring glycolysis at LOS and PPP at HOS, appears consolidated by the long series of reported papers[39,40]. Many experimental data confirm that this O_2-linked RBC modulation is played at the level of the RBC membrane involving the quaternary state of band 3[41]. Although it is difficult to evaluate the role played by the different factors, it seems probable that several aspects are crucial for this mechanism:

1. The oxygenation state of Hb;
2. The phosphorylation level of the N-terminal domain of band 3.

Thus, through this review we hope to stimulate further research in this important area, in order to give new insight on the molecular events that regulate phosphorylation of band 3, and its connection with red blood cell metabolism and function.

Keywords: haemoglobin; band 3; glycolytic enzymes; erythrocyte.

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