

Band 3 Protein Function in Teleost Fish Erythrocytes : Effect of Oxygenation-Deoxygenation

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Summary

During vertebrate evolution, structural changes in red blood cells (rbc) and hemoglobin (Hb), have probably resulted in the importance of blood carbon dioxide transport. The chloride/bicarbonate exchange across the rbc membrane, which is an integral part of the blood CO₂ transport process in vertebrates, has been examined on two different species of teleost fish, *Euthynnus alletteratus* and *Thunnus thynnus*, at several oxygenation states of erythrocyte HOS (high-oxygenation state, about 90 % of saturation) and LOS (low-oxygenation state, about 15 % of saturation). The results were compared with those observed in human rbc under the same experimental conditions and with the chicken (*Gallus gallus*) erythrocytes which have particular modifications at the N-terminus of the band 3 protein (B3). In fish the kinetic measurements have shown a different anion transport in several oxygenation states of the erythrocytes, indicating that also at lower levels of vertebrate evolution there exists a modulation of the anionic flow affected by oxygen. The functional correlation of anion transport to changes of parts of the hemoglobin sequence responsible for alterations in the interactions with the cytoplasmic domain of band 3 protein (cdb3) allowed us to suggest a hypothesis about fish physiology. The highest values of kinetic measurements observed in fish have been attributed to the metabolic need of the rbc in response to the removal of CO₂ that in teleosts is also of endogenous origin.

Key words.

Erythrocyte • Hemoglobin • Band 3 protein • Anion transport • Oxygenation-deoxygenation cycle.

Introduction

The complex function of the transport of oxygen and carbon dioxide in blood is closely associated with the electrolytic and acid-base equilibrium of the red blood cell, markedly depending on membrane permeability and the function of proteins contained in it. (Thomas *et al.*, 1998)

Moreover, various physical and functional characteristics of the erythrocyte (including glycolysis, pentose-phosphate cycle, ion fluxes and cell deformability) show a strong dependence on the state of oxygenation of hemoglobin, whose molecular mechanism involves the interaction with another abundant protein present at the membrane, viz. the band 3 protein.

While the Hb carries out the function of oxygen transport from lungs or gills to the tissues, the band 3 protein carries out the electrically neutral exchange of chloride and bicarbonate anions between the inside and the outside of the red blood cell. The active form of the band 3 protein is a dimer in which each monomer, represented by 920 amino acids is subdivided in two fundamental regions: a hydrophobic region that crosses the membrane with 12–14 α -helices and a cytoplasmic hydrophilic region (cdb3) distinct in its turn in two domains, N-terminal and C-terminal. The integral membrane domain represents the protein portion involved in the function of an ion channel; the cytoplasmic domain connects binding with various intraerythrocytic proteins, among them the cytoskeletal proteins (ankyrin, band 4,1 and band 4,2) and carbonate dehydratase to the extreme C-terminus (Bruce *et al.* 2003), the glycolytic enzymes (Low 1986, Harris *et al.* 1990, Low *et al.* 1993) and hemoglobin (Cassoly 1983, Chetrite *et al.* 1985) to the N-terminus.

In particular Hb binds to cdb3 through β -chains; this binding is mostly of electrostatic nature and is influenced by the state of oxygenation of the erythrocyte. The interaction causes a sort of bivalent modulation, decreasing the hemoglobin affinity for oxygen and changing the $\text{HCO}_3^-/\text{Cl}^-$ flux, probably due to a kind of steric hindrance exercised by Hb on the intermembrane channel (Galtieri *et al.* 2002). The binding between band 3 and hemoglobin takes place at the level of the first extremely acid 11 amino acid residues of the N-terminal portion of the band 3 cytoplasmic domain. This portion fits perfectly into the central cavity of the hemoglobin molecule at the level of 2,3-biphosphoglycerate (BPG) binding cleft involving all the basic residues responsible for binding with phosphate (that is Val1, His2, His143 and Lys 82) beyond to Arg104 residue characteristic for the binding with cdb3. Some glycolytic enzymes interact also with the N-terminal part of the band 3 and their binding causes a kind of competition with the Hb for the same site. Since band 3 coordinates the transmembrane anion exchange ($\text{HCO}_3^-/\text{Cl}^-$) and the glycolytic enzymes govern the metabolic pathway in the erythrocyte, it follows that Hb can work like a transducer, through its T-R transition, modulating the metabolism of the red blood cell and the anion exchange in an oxygen-dependent way (Giardina *et al.* 1995, Galtieri *et al.* 2002).

The question then naturally arises whether vertebrate Hbs at the lowest evolutionary level can have evolved an oxygen-dependent affinity for their respective cdb3, assuming that in the erythrocyte the evolution of a regulatory mechanism modulating some fundamental functions is desirable and predictable. Arguments against such hypothesis include the fact that in vertebrates the object of study is the N-terminus of band 3 sequence is not highly conserved, while supporting data indicate that all the cdb3 known are polyanionic and that many deoxygenated Hb (deoxyHb) of vertebrates show high affinity for polyanions, such as BPG, IPP or ATP (Weber *et al.* 2004).

Since it is known that many evolutionary changes are the result of amino acid replacements in biologically important proteins, starting with the premise that the erythrocytes carry out anion exchange through band 3 and that their Hbs as well as the type of organic phosphates in the red blood cell are different, we performed a kinetic study of $\text{HCO}_3^-/\text{Cl}^-$ exchange. The aim of our work is to verify if in species with brachial respiration (first level of vertebrate evolution), with nucleated red blood cells and an oxygen-dependent metabolism, the function of band 3 is modulated by the pressure of oxygen as we have shown earlier (Galtieri *et al.* 2002) in species with pulmonary respiration (last stage of vertebrate evolution), with anucleate erythrocytes and with metabolism involving the central carbohydrates in an absolutely oxygen-independent way. Moreover, we wish to show whether the energetic (ATP) or the reducing (NADPH) metabolic response in a fish red blood cell, remarkably more complex (structurally and functionally) than that of fully developed vertebrate (mammalian) one are more important. In fact, in fish erythrocytes oxygen is not only an “active” transit molecule, but also the final protagonist with an effective use in electron transport (producing H_2O and ATP) and that is fed from the Krebs cycle generating endogenous carbon dioxide, which is the “substrate” of the band 3 protein.

Methods

Material

All reagents were from Sigma-Aldrich (St. Louis, MO, USA). Human erythrocyte samples were collected from informed healthy volunteers aged 30-50 years under the declaration that they had avoided any drug treatment at least one week before sample collection. Fish and chicken blood was obtained from local fish farm and slaughterhouse, respectively.

Preparation of red blood cells

Heparinized blood samples were washed three times with iso-osmotic NaCl solution. During washings the white blood cells were discarded from the pellet. After washing the red blood cells were resuspended (hematocrit 3%) in the incubation buffer (35 mM Na_2SO_4 , 90 mM NaCl, 25 mM

HEPES [*N*-(2-hydroxyethyl)-piperazine-*N*¹-2-ethanesulfonic acid], 1.5 mM MgCl₂), adjusted to pH 7.4 or 7.3 and 310 ± 20 mOsmol per kg, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco, Kyoto, Japan).

In experiments performed on deoxygenated erythrocytes, samples were submitted to cycles of deoxygenation in vacuo and nitrogen (ultrapure) saturation at a pressure of 760 Torr. This treatment allowed us to obtain different levels of deoxygenation (from 15 % to 90 %), which were checked by determining hemoglobin saturation spectrophotometrically (Beckman DU 70 spectrophotometer) using the millimolar absorptivities reported by Zijlstra *et al.* (1991).

The buffer used to prepare deoxygenated erythrocytes was by 0.1 pH unit lower than that used for oxygenated erythrocytes, in order to compensate for the Haldane effect occurring during the deoxygenation step (Labotka *et al.* 1984). Thus, after the deoxygenation procedure, the oxygenated and deoxygenated samples had an external pH differing by no more than 0.03 pH units.

Kinetic measurements

Cells were incubated in the above incubation buffer at 25°C, under different experimental conditions. At several time intervals, 10 micromoles of the stopping medium SITS (4-acetamido-4¹-isothiocyano stilbene-2,2¹-disulfonic acid) were added to each test tube containing 5 ml of the red blood cell suspension. Cells were then separated from the incubation medium by centrifugation (J2-HS Centrifuge, Beckman, Palo Alto, CA, USA) and washed three times at 4 °C with a sulfate-free medium to remove sulfate anions trapped outside. After the last washing packed cells were lysed with perchloric acid (4 %) and distilled water (2.8 ml final volume). The lysates were centrifuged for 10 min at 4000 × *g* (4 °C) and membranes were separated from the supernatant. Sulfate ions were precipitated from the supernatant by adding 1 ml of glycerol/distilled water mixture (1:1, V/V), 0.5 ml of 4 M NaCl and 1M HCl, 0.5 ml of 1.23 M BaCl₂·2H₂O in order to obtain a homogeneous barium sulfate precipitate. The absorbance of this suspension was measured at 350–425 nm.

Using a calibrated standard curve, obtained by measuring the absorbance of suspensions obtained from solutions containing known sulfate amounts, the sulfate concentration was determined (Romano *et al.* 1998). Experimental data of sulfate concentration as a function of time incubation were analyzed by best fitting procedures according to the following equation:

$$C(t) = C_{\infty} (1 - e^{-kt})$$

where $C(t)$ represents sulfate concentration at time t , C_{∞} the intracellular sulfate concentration at equilibrium, and k the rate constant of sulfate influx.

Results

The activity of anion exchange operating from the band 3 appears in a marked manner in the oxygenation state of the erythrocyte, as shown in Fig.1, where it is represented as the kinetics of flux measured in the human red blood cells at several oxygen pressures. Averages of rate constant values determined for the sulfate influx in the oxygenated erythrocytes (HOS, high-oxygenation state, about 90 % saturation) and deoxygenated ones (LOS, low-oxygenation state, about 15 % saturation) indicate a remarkable increase of flux when the red blood cell passes from LOS to HOS state (rate constant from 0.005 to 0.012 min⁻¹).

In order to exclude the contribution of other transport systems influenced by the oxygen pressure, we carried out a set of experiments on red cells pretreated with SITS, a specific inhibitor of band 3 activity. Under these experimental conditions no sulfate transport was observed.

Since teleost fish possess band 3 protein copies like that found in human anucleate erythrocytes and nothing is known about the oxygen influence on the antiport Cl⁻/HCO₃⁻ dynamics; we performed a series of experiments on the kinetics of the anionic flux in the red blood cells of two teleost fish species (northern blue-finned tuna *Thunnus thynnus* and Atlantic little tuna *Euthynnus alletteratus*), belonging to the family Scombridae because nucleated fish erythrocytes different mechanisms of ion transport are highly sensitive to the degree of oxygenation and to the Hb conformation (Motais *et al.* 1987, Jensen 1990) and that we already know the functional property of the hemoglobins from the two selected species (data not published). The results are shown in Fig.2 where it is possible to observe that the rate of sulfate influx inside the rbc is similar in both species under conditions of high oxygen saturation (rate constant 0.242 min⁻¹ for *T. thynnus* and 0.286 min⁻¹ for *E. alletteratus*) or of low oxygen saturation (rate constant 0.148 min⁻¹ and 0.115 min⁻¹, respectively). From a direct comparison with the anion transport kinetics in human red blood cells it should be noted that the anion flux is remarkably higher and that HOS and LOS erythrocyte states, also in this case, influence the band 3 protein activity although in a less marked way. In this respect, the results shown in Table 1 in human rbc point to a decrease (approximately 60 %) of anion exchange when the experiments were performed at low oxygen pressure, while in fish red blood cells the passage from the HOS to LOS state brought about a smaller flux reduction (approximately 40 %).

This different influence of oxygen on the band 3 protein functionality could be due to the presence in fish erythrocytes of an Hb partially replaced in the binding pocket with cdb3. It would result in the weakness of the Hb-B3 bond which would promote a lower modulation of the oxygen transporter molecule on the band 3 activity and therefore a lower sensibility of it to the oxygen pressure.

In order to show the effective role of the Hb-cdb3 interaction we carried out experiments on sulfate transport kinetics in chicken erythrocytes, which are known to possess a modified cdb3, lacking the site of glycolytic enzymes (Jay 1983). The experiments performed on the sulfate transport in this case did not indicate meaningful differences at the various oxygen saturations (0.07 min^{-1}).

Discussion

We showed here that in the erythrocytes of two teleost fish *Thunnus thynnus* and *Euthynnus alletteratus* the sulfate transport carried out by band 3 protein is closely linked to oxygen pressure. The anion flux through the membrane was decreased of about 40 % in both species in LOS erythrocytes (Fig. 2, Table 1); a similar result (decrease of approximately 60 %) was obtained in human red blood cells under the same experimental conditions (Fig. 1, Table 1). This finding suggests the presence of a regulatory mechanism for the anion transport at the early stages of vertebrate evolution, like that observed in the fully developed vertebrates. We assumed that the different activity of band 3 in HOS and LOS state was connected with the conformational transition of Hb (T-R) due to oxygen pressure. Although direct evidence of any functional communication between the cdb3 binding site and the anion transport site cannot be established, all data obtained in this work can be rationalized on the basis that cdb3/deoxy-Hb binding influences the anion transport more than the cdb3/oxy-Hb does (Walder *et al.* 1984). Since T-Hb has a higher affinity for cdb3 than R-Hb, a gradual increase of Hb bound to band 3 protein and consequently an increment of structural hindrance would take place on passing from HOS to LOS. The hindrance caused by deoxy-Hb on the band 3 channel justifies the reduction of the anion transport found in the deoxygenated erythrocytes. It is thus probable that the small difference of the kinetics of chloride exchange observed at several oxygenation states in fish red blood cells compared to that found in human ones, is due to a different interaction between Hb and band 3, as a consequence of a series of amino acid replacements in teleost fish Hb (Weber 2000). In fact, the primary structure of these Hbs is characterized by a reduced presence of basic residues compared with human Hb, just to the level of the binding site with cdb3. In particular, both β -chains show replacements Glu/Asp β 2 \rightarrow His and Arg β 143 \rightarrow His involved in the binding with BPG, and the replacement Gln \rightarrow Pro β 5 (Rodewald *et al.* 1987). The replacement in β 2 brings about the disappearance of two positive charges in the anionic pocket that, if it is stereochemically adapted to the interaction with ATP (physiological cofactor of fish, with less negative charges than BPG), weakens the positive charge cluster present on the Hb molecule, useful in the electrostatic interaction with cdb3. Moreover, the affinity constant between cdb3 and teleost Hb is further reduced by the β 5 replacement where a glutamine residue is changed to proline. This substitution that was evidenced also in many other species, such

as whale, bat and reindeer, could cause a misfolding of the α -helix, with a consequent variation of the binding stoichiometry between cdb3 and Hb (Giardina *et al.* 1990, Corda *et al.* 2003).

All these factors would contribute to weakening the binding between fish Hb and cdb3, so the reduced difference of the anion exchange through the membrane between oxygenated and deoxygenated rbc, could be the consequence of a reducing and easily removable hindrance of the deoxy-Hb on the anionic channel. Arguments in support of this view rely on experiments performed with chicken erythrocytes, in which no differences in the band 3 activity between the HOS and LOS states were found. This finding should be linked to the absence in chicken cdb3 of the binding site for glycolytic enzymes and, therefore, to missing interaction between deoxy-Hb and the band 3 protein.

From a physiological and evolutionary point of view the differences found between fish and human $\text{HCO}_3^-/\text{Cl}^-$ exchange could be related to the energetic requirements of their red blood cells. In fact, human erythrocytes lacking mitochondria, derive their energy from the anaerobic metabolism nearly completely dependent on glycolysis and the pentose phosphate shunt. This involves the need of a fine and efficient metabolic modulation of glucose-6-phosphate carried out through interaction between hemoglobin and band 3 protein. The bird and ectothermic vertebrate erythrocytes, in contrast, rich in mitochondria and mainly oriented toward aerobic metabolism, can respond to their metabolic requirements through a less marked Hb-cdb3 interaction (Wang *et al.* 1994). In this regard, it is significant to note that the weak Hb-cdb3 bond in teleost fish rbc allows an early activation of the pentose phosphate way toward lower oxygen concentrations than in human erythrocytes. In fact, the glycolytic enzyme binding sites will then slow down the glycolytic pathway and glucose-6-phosphate will enter in the pentose phosphate pathway. Consequently, the stronger production of reducing power will also allow a more effective cell defence against the free radicals derived from the direct use of oxygen through the oxidative phosphorylation too. Likewise, the reducing power is necessary to increase the typical biosynthetic ways of a nucleate erythrocyte that can much more advantageously use aerobic metabolism with a higher production of adenosine triphosphate (18 times) than the anucleate one. The reduced glucose-6-phosphate use in the Embden-Meyerhof pathway does not decrease the oxygen release because the physiological modulator of fish hemoglobin is ATP, an organic phosphate more easily produced from the aerobic metabolism. Added to this metabolic picture we found: an increase of anionic exchange activity across the band 3 protein, certainly due to the presence in the cell of an aerobic metabolism. The high $\text{HCO}_3^-/\text{Cl}^-$ flux observed in teleosts could be correlated to the need for a more efficient transport of CO_2 that is not only of exogenous origin (tissue metabolism) but is also produced in an endogenous way by aerobic metabolism of carbon dioxide. In fact, it is known that there is an

increased presence of CO₂ in fish erythrocytes (compared to anucleate rbc) partly due to exogenous origin but mainly due to the efficiency of the Krebs cycle, that leads to the necessity to expel the excess CO₂, which is confirmed by the stronger activity of band 3 protein.

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Reprint requests

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Table 1. Rates of sulfate transport measured in oxygenated and deoxygenated human and fish red blood cells.

Species	Rate constant (min ⁻¹)	
	Oxygenated	deoxygenated
<i>Homo sapiens</i>	0.0120	0.0052
<i>Thunnus thynnus</i>	0.242	0.148
<i>Euthynnus alletteratus</i>	0.286	0.115

Fig 1. Rates of sulfate influx determined in human erythrocytes under different oxygen pressure. For experimental conditions see Methods.

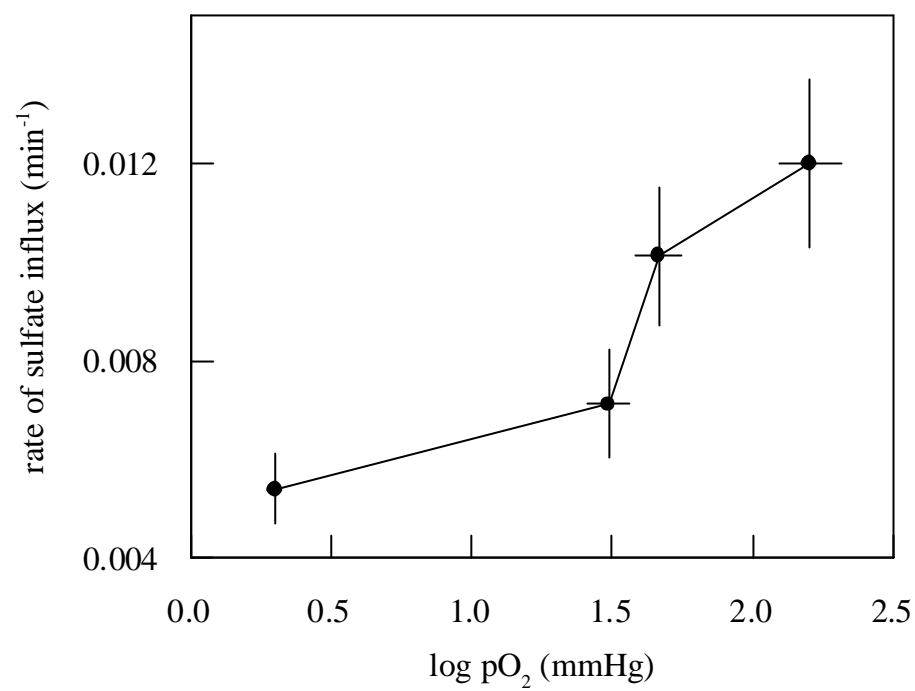


Fig. 2. Sulfate concentration (mg/l) determined in red blood cell lysates at different incubation times. Results of a typical experiment performed by incubating *Thunnus thynnus* (closed symbols) and *Euthynnus alletteratus* (open symbols) erythrocytes under high (triangles) and low (circles) oxygen pressure. Curves were obtained by fitting experimental data with $C(t) = C_{\infty} (1 - e^{-kt})$. See Methods for further experimental data.

