ELSEVIER

Biochimica et Biophysica Acta 1564 (2002) 214–218

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Band-3 protein function in human erythrocytes: effect of oxygenation–deoxygenation

Antonio Galtieri [a], Ester Tellone [a], Leonardo Romano [b], Francesco Misiti [c], Ersilia Bellocco [a], Silvana Ficarra [a], Annamaria Russo [a], Domenica Di Rosa [d], Massimo Castagnola [c,e], Bruno Giardina [c,e], Irene Messana [e,f,*]

[a] *Dipartimento di Chimica Organica e Biologica, Università di Messina, Messina, Italy*
[b] *Istituto di Fisiologia Generale, Università di Messina, Messina, Italy*
[c] *Istituto di Biochimica e Biochimica Clinica della Facoltà di Medicina e Chirurgia dell'Università Cattolica, Roma, Italy*
[d] *Virologia-Ospedale Azienda Papardo, Messina, Italy*
[e] *Centro di Studio per la Chimica dei Recettori e delle Molecole Biologicamente Attive, C.N.R., Roma, Italy*
[f] *Dipartimento di Scienze Applicate ai Biosistemi, Università di Cagliari, Cagliari, Italy*

Received 23 October 2001; received in revised form 27 February 2002; accepted 22 April 2002

Abstract

Sulfate transport by band-3 protein in adult human erythrocytes was shown to be modulated by oxygen pressure. In particular, a higher transport activity was measured under high oxygen pressure than at low one (0.0242 ± 0.0073 vs. 0.0074 ± 0.0010 min^{-1}). Other factors, such as magnesium ions and orthovanadate, which can indirectly affect the binding properties of the cytoplasmic domain of band 3 (cdb3), influence significantly the anion exchanger activity. No effect of oxygen pressure on sulfate transport was found in chicken erythrocytes, which may be related to their lacking the cdb3 binding site. These findings are fully consistent with a molecular mechanism where the oxygen-linked transition of hemoglobin (T → R) could play a key role in the regulation of anion exchanger activity.

Keywords: Erythrocyte; Sulfate transport; Band-3 protein; Oxygenation–deoxygenation cycle; Hemoglobin

1. Introduction

The main role of erythrocytes is to mediate the oxygen–carbon dioxide exchange between tissues and lungs. Red cells fulfil this requirement, during their life-span, fluctuating between two limiting states, high-oxygenation state (HOS erythrocyte) and a low-oxygenation one (LOS erythrocyte).

HOS and LOS limiting states are in some way functionally and molecularly associated with the corresponding R and T states of hemoglobin. Accordingly, the free energy coupled to the T → R transition of Hb may be utilised for a change of the structural and metabolic erythrocyte demands. In this respect, many studies have shown that metabolism, membrane fluidity, concentration of ions and intermediates, and activity of many red cell transport proteins are substantially different in HOS and LOS erythrocytes, in such a way that pO_2 seems to represent a specific and selective signal [1–3]. The molecular events, which link the T → R transition and HOS and LOS general properties, are very complex and they cannot be easily elucidated. Several of these events are probably confined to the membrane level, where band-3 protein probably plays a pivotal role. The active form of this protein, present in a quantity of about 1.2×10^6 copies per red cell, consists of a dimer with a membrane-spanning domain (55 kDa), responsible for anion exchange across the membrane, and a cytoplasmic domain (cdb3, 43 kDa) displaying modulator functions [4,5]. In fact, cdb3 binds cytoskeletal proteins (band 2.1 protein or ankyrin) [6] and several glycolytic enzymes [7], such as aldolase [8], phosphofructokinase [9], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [10], lactate dehydrogenase [11] and the protein tyrosine kinase ($p72^{syk}$) [12]. In addition, cdb3 binds to hemoglobin [13–15]. This interac-

* Corresponding author. Dipartimento di Scienze Applicate ai Biosistemi, Università di Cagliari, Strada Provinciale Monserrato-Sestu Km 0.7, 09042 Monserrato, Cagliari, Italy. Tel.: +39-0706754-548; fax: +39-0706754-523.

E-mail address: i.messana@uniserv.ccr.rm.cnr.it (I. Messana).

0005-2736/02/$ - see front matter
PII: S0005-2736(02)00454-6

tion is predominantly electrostatic in nature and is influenced by the oxygenation state of the red cell, since deoxyhemoglobin (T-state) binds the cdb3 more tightly than oxygenated hemoglobin (R-state) does ($K_{ass} \approx 10^4$ and 10^2 M^{-1}, respectively) [16]. It has been reported that GAPDH and hemoglobin bind to residues 1–11 of cdb3, while aldolase binds to both residues 1–11 and 13–31 [7]. Experimental data support the hypothesis that binding of deoxyhemoglobin to cdb3 could be responsible for the oxygen-dependent modulation of erythrocyte glucose metabolism [17].

Within this emerging scheme, hemoglobin appears to display, besides the basic function of oxygen transport, several other biological functions which are driven by the oxygen-linked conformational transition and whose relative importance, in the economy of the cell and of the organism, is not easy to qualify. The literature provides no clear information concerning the effects of O_2 pressure on the transport activity of band 3 [1,18]. Thus, with the aim to increase the experimental information concerning the interplay existing between the erythrocyte oxygenation–deoxygenation cycle and the functional properties of the human erythrocyte anion exchanger, we investigated the transport properties of band-3 protein under different red cell oxygenation states.

2. Materials and methods

2.1. 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 1 week before sample collection. Chicken blood was obtained from local slaughterhouse.

2.2. Preparation of red blood cells

Heparinized blood samples were washed three times with 166 mM 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];+/− 4 mM $MgCl_2$, adjusted to pH 7.4 or 7.3 and 310 ± 20 mOsm/kg, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco, Kyoto, Japan).

In experiments performed on deoxygenated erythrocytes, samples were submitted to cycles of in vacuum deoxygenation and nitrogen (ultrapure) saturation at a pressure of 760 Torr. This treatment allowed us to obtain different levels of deoxygenation (from 15% up to 90%), which were checked by determining hemoglobin saturation spectrophotometrically (Beckman DU 70 spectrophotometer) using the millimolar absorptivities reported by Zijlstra et al. [19].

The buffer used to prepare deoxygenated erythrocytes was 0.1 pH unit lower than that used for oxygenated erythrocytes, in order to compensate the Haldane effect occurring during the deoxygenation step [20]. Thus, after the deoxygenation procedure, the oxygenated and deoxygenated samples had an external pH no more different than 0.03 pH units.

2.3. Kinetic measurements

Cells were incubated in the above incubation buffer at 25 °C, under different experimental conditions. At several time intervals, 10 μmol of the stopping medium SITS (4-acetamido-4^1-isothiocyanostilbene-2,2^1-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 SO_4^{2-}-free medium to remove SO_4^{2-} trapped outside. After the last washing packed cells were lysed with perchloric acid (4%) and distilled water (2.8 ml final volume). Lysates were centrifuged for 10 min at $4000 \times g$ (4 °C) and membranes were separated from the supernatant. SO_4^{2-} 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 1 M HCl, 0.5 ml of 1.23 M $BaCl_2 \cdot 2H_2O$ 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 SO_4^{2-} amounts, the SO_4^{2-} concentration was determined [21]. Experimental data of sulfate concentration as a function of time incubation were analysed by best fitting procedures according to the following equation:

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

where $C(t)$ represents SO_4^{2-} concentration at time t, C_∞ intracellular SO_4^{2-} concentration at equilibrium, and k the rate constant of SO_4^{2-} influx.

2.4. Statistical analysis

Differences were analysed with a two-tailed Student's t test for unpaired data. The results are expressed as means ± S.D. Probabilities less than or equal to 0.05 were considered as statistically significant.

3. Results

Fig. 1 reports, as an example, the rate of SO_4^{2-} transport in human oxygenated (HOS, about 90% of saturation) and deoxygenated (LOS, about 15% of saturation) erythrocytes

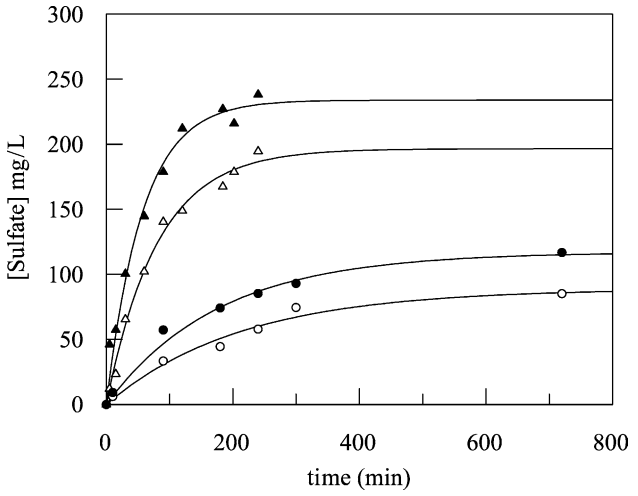

Fig. 1. SO_4^{2-} concentration (mg/l) determined in red blood cell lysates at different incubation times. Results of a typical experiment performed by incubating erythrocytes in a medium containing 4 mmol/l Mg^{2+} under high (closed triangles) and low (closed circles) oxygen pressure, and in a medium without Mg^{2+} under high (open triangles) and low oxygen pressure (open circles). Curves were obtained by fitting experimental data with the equation: $C(t)=C_\infty(1-e^{-kt})$. See Materials and methods for further experimental details.

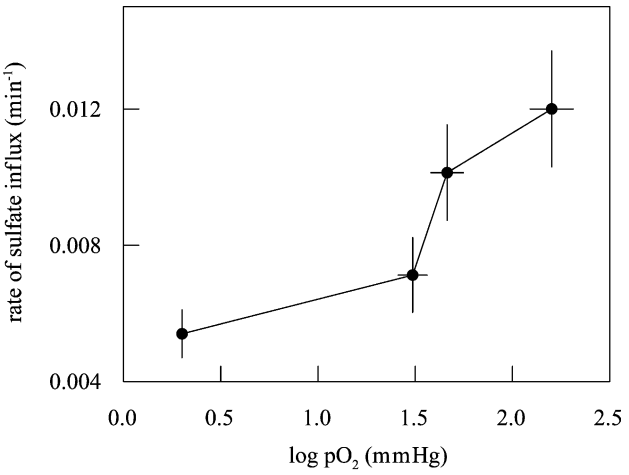

Fig. 2. Rates of SO_4^{2-} influx determined in human erythrocytes under different oxygen pressure. For experimental conditions see Materials and methods. Rates are reported in min^{-1} and pO_2 values in mmHg.

with and without 4 mM $MgCl_2$. Since it is known that oxygen affects the activity of several transport systems in erythrocytes [1], a set of experiments was performed on red cells pretreated with SITS, a specific inhibitor of band 3 activity. Under this experimental condition no SO_4^{2-} transport was observed; hence, we excluded the contribution of other transport systems to our observations. Free magnesium ions during the red cell oxygenation–deoxygenation cycle competes with hemoglobin for the interaction with 2,3-BPG and ATP [22]. Indeed, we observed that sulfate transport decreased when experiments were performed in the absence of magnesium ions. However, the differences between HOS and LOS erythrocytes were maintained, as shown in Table 1.

Table 1
Rates of SO_4^{2-} transport measured in oxygenated and deoxygenated adult human red blood cells with and without magnesium ions and orthovanadate ($P<0.05$ was considered significant)

	Rate constant (min^{-1})	Rate constant (min^{-1})	P
Medium composition	oxygenated	deoxygenated	HOS vs. LOS
Control	0.0120 ± 0.0026 ($N=4$)	0.0052 ± 0.0006 ($N=5$)	0.0007
4 mM Mg^{2+}	0.0242 ± 0.0073 ($N=6$)	0.0074 ± 0.0010 ($N=4$)	0.0021
Orthovanadate	0.0184 ± 0.0037 ($N=4$)	0.0055 ± 0.0014 ($N=4$)	0.0006
P (control vs. 4 mM Mg^{2+})	0.013	0.005	
P (control vs. orthovanadate)	0.029	n.s.	

The mean rate constant values determined for SO_4^{2-} influx on HOS and LOS erythrocytes, reported in Table 1, indicated that the band 3 transport works about three times faster in HOS erythrocytes than in LOS. This finding induced us to investigate the transport under different oxygen pressures. Fig. 2 shows the dependence of SO_4^{2-} influx rate on log pO_2.

It is well known that orthovanadate affects cell protein phosphorylation, including cdb3 [23,24]; we performed a set of experiments in the presence of orthovanadate. The results are reported on Table 1 and they indicate that whereas in HOS erythrocytes orthovanadate clearly increases the activity of band 3, differences between treated and untreated cells are not detectable in LOS erythrocytes.

A further possibility to clarify whether the cdb3 and Hb interaction plays a role in band-3 anionic transport concerns the use of erythrocytes with a modified cdb3. In this respect, it is well known that band 3 of chicken erythrocytes lacks the site of glycolytic enzyme binding. Hence, we measured SO_4^{2-} transport in HOS and LOS chicken erythrocytes. In this case the rate constant values determined were not significantly different (0.07 ± 0.019 min^{-1}).

4. Discussion

To investigate the dependence of band-3 transport on oxygen pressure and on Hb saturation, a selective measurement tool is required. In this respect, whereas the majority of Cl^- cotransporters and antiports are Cl^- selective, band 3 transporter can exchange different anions, even if at different rates. The use of SO_4^{2-} transport to monitor band-3 activity offers the advantage that the exchange time is slow enough to organise a relatively simple experimental proto-

col. Exchange of SO_4^{2-}/Cl^- is not electrogenic since SO_4^{2-} influx is accompanied by H^+ cotransport [25]. Moreover, the virtual absence of SO_4^{2-} within the erythrocyte ensures that intracellular SO_4^{2-} determinations are essentially indicative of the anion uptake [21]. The specificity of this approach was confirmed by the practically absent SO_4^{2-} transport observed in SITS-treated erythrocytes.

The results of this study demonstrate that in human erythrocytes SO_4^{2-} transport by band-3 is oxygen-dependent. It was reduced by about 50% (Table 1, Fig. 1) in human LOS erythrocytes. A similar result was obtained in the absence of Mg^{2+} (Table 1, Fig. 1), suggesting that intracellular concentration of Mg^{2+} was not involved in the observed phenomenon. We assumed that the different activity of band 3 in HOS and LOS state was connected with the oxygen-linked conformational transition of hemoglobin ($T \rightarrow R$). The results shown in Fig. 2 strongly support this assumption. In fact, the curve that describes band 3 activity as a function of pO_2 has a shape similar to the oxygen binding curve of human Hb. In the LOS state, the activity of free Cl^- is lower, as consequence of its binding to Hb. Since SO_4^{2-} influx across band 3 is coupled with Cl^- efflux, it might be argued that the observed difference in band 3 activity between HOS and LOS states is induced by the decreased intracellular activity of free Cl^-. We believe that the reduction of Cl^- activity is not sufficient to justify the low SO_4^{2-} exchange observed in LOS erythrocyte, by considering up to two binding sites for Cl^- for each Hb tetramer. Other important events that occur at the level of cdb3 are affected by the HOS–LOS transition. Indeed, recent studies have demonstrated that the competition of deoxy-Hb and phosphofructokinase for cdb3 can exploit a modulation of erythrocyte metabolism [2,17]. Long-range conformational changes in cdb3 structure, consequent upon Hb binding, were already suggested by the detection of a decrease of deoxy-Hb binding to cdb3 in DIDS (4-4^1-bis (isothiocyano)-2,2^1-stilbenedisulfonic acid)-treated erythrocytes [26]. Although direct evidence of any functional communication between the cdb3 binding site and the anion transport site cannot be established, all data obtained here can be rationalised on the basis of cdb3/deoxy-Hb binding. Due to the higher affinity of T-Hb for cdb3 with respect to R-Hb [16], a gradual increase of bound hemoglobin and, consequently, an increase of structural hindrance occur on going from HOS to LOS. Experiments performed in the presence of orthovanadate demonstrated an increased activity of band 3 transport only in HOS erythrocytes. Orthovanadate has an indirect effect on band 3 phosphorylation [23,24] and, as suggested by Low et al. [27], causes an impairment of glycolytic enzyme binding to cdb3. Thus, the higher activity of band 3 in the HOS state may be connected with the lower percentage of bound enzymes. On the other hand, few reports exist on the effect of phosphorylation on Hb affinity for cdb3 [28]. It is known that Hb binds to cdb3 at the organic phosphate binding site within the two β-chains. We assumed that additional negative charges of cdb3, due to the phosphorylation of the tyrosine residues, did not affect Hb binding.

In experiments performed by the continuous flow tube method, Jensen and Brahm [18] found significant interspecies variation in Cl^- transport kinetics between fish red blood cells. In contrast, in all the species examined, including human erythrocytes, they did not find any difference in the Cl^- exchange rate between oxygenated and deoxygenated cells. The different experimental approach utilised in our study and the different pH range investigated may explain these discordant data. In fact, the use of radioactive Cl^- in the continuous flow tube method could provide a measure of the activity of different cotransporters and exchangers, the properties of which can be oppositely affected by the degree of Hb oxygenation.

In addition, we did not observe a different activity of band 3 in HOS and LOS adult chicken erythrocytes. This finding should be linked to the absence in chicken cdb3 of the binding site for glycolytic enzymes [29].

The increased anion exchange transport across band 3 observed in HOS erythrocytes may be considered as functional to general red cell requirements. Indeed, red blood cell in vivo has a higher requirement of Cl^-/HCO_3^- exchange activity during the lung oxygen loading.

References

[1] J.S. Gibson, A.R. Cossins, J.C. Ellory, J. Exp. Biol. 203 (2000) 1395–1407.
[2] B. Giardina, I. Messana, R. Scatena, M. Castagnola, Crit. Rev. Biochem. Mol. Biol. 30 (1995) 165–196.
[3] S. Tuvia, S. Levin, R. Korenstein, Biophys. J. 63 (1992) 599–602.
[4] H. Passow, Rev. Physiol., Biochem. Pharmacol. 103 (1986) 61–203.
[5] D. Jay, L. Cantley, Annu. Rev. Biochem. 55 (1986) 511–538.
[6] V. Bennett, Annu. Rev. Biochem. 54 (1985) 273–304.
[7] P.S. Low, Biochim. Biophys. Acta 864 (1986) 145–167.
[8] S.N. Murthy, T. Liu, R.K. Kaul, H. Kohler, T.L. Steck, J. Biol. Chem. 256 (1981) 11203–11208.
[9] R. Higashi, C.S. Richards, K. Uyeda, J. Biol. Chem. 254 (1979) 9542–9550.
[10] H.J. Kliman, T.L. Steck, J. Biol. Chem. 255 (1980) 6314–6321.
[11] S.J. Harris, D.J. Winzor, Biochim. Biophys. Acta 1038 (1990) 306–314.
[12] M.L. Harrison, C.C. Isaacson, D.L. Burg, R.L. Geahlen, P.S. Low, J. Biol. Chem. 269 (1994) 955–959.
[13] J.M. Salhany, Erythrocyte Band 3 Protein, CRC Press, Boca Raton, FL, 1990.
[14] R. Cassoly, J. Biol. Chem. 258 (1983) 3859–3864.
[15] G. Chétrite, R. Cassoly, J. Mol. Biol. 185 (1985) 639–644.
[16] J.A. Walder, R. Chatterjee, T.L. Steck, P.S. Low, G.F. Musso, E.T. Kaiser, P.H. Rogers, A. Arnone, J. Biol. Chem. 259 (1984) 10238–10246.
[17] I. Messana, M. Orlando, L. Cassiano, L. Pennacchietti, C. Zuppi, M. Castagnola, B. Giardina, FEBS Lett. 390 (1996) 25–28.
[18] F.B. Jensen, J. Brahm, J. Exp. Biol. 198 (1995) 2237–2244.
[19] W.G. Zijlstra, A. Buursma, W.P. Meeuwsen-van der Roest, Clin. Chem. 37 (1991) 1633–1638.
[20] R.J. Labotka, Biochemistry 23 (1984) 5549–5555.
[21] L. Romano, D. Peritore, E. Simone, A. Sidoti, F. Trischitta, P. Romano, Cell. Mol. Biol. 44 (1998) 351–355.
[22] P.J. Mulquiney, P.W. Kuchel, Eur. J. Biochem. 245 (1997) 71–83.

[23] A.M. Brunati, L. Bordin, G. Clari, P. James, M. Quadroni, E. Baritono, L.A. Pinna, A. Donella-Deana, Blood 96 (2000) 1550–1557.
[24] L. Bordin, G. Clari, I. Moro, F. Dalla Vecchia, V. Moret, Biochem. Biophys. Res. Commun. 213 (1995) 249–257.
[25] M.L. Jennings, J. Membr. Biol. 28 (1976) 187–205.
[26] J.M. Salhany, K.A. Cordes, E.D. Gaines, Biochemistry 19 (1980) 1447–1454.
[27] P.S. Low, A. Kiyatkin, Q. Li, M.L. Harrison, Protoplasma 184 (1995) 196–202.
[28] P.S. Low, D.P. Allen, T.F. Zioncheck, P. Chari, B.M. Willardson, R.L. Geahlen, M.L. Harrison, J. Biol. Chem. 262 (1987) 4592–4596.
[29] D.G. Jay, J. Biol. Chem. 258 (1983) 9431–9436.