Trakia Journal of Sciences, Vol. 8, Suppl. 2, pp 6-11, 2010

Available online at:
http://www.uni-sz.bg

ISSN 1313-7050 (print)
ISSN 1313-3551 (online)

BAND 3 PROTEIN INVOLVEMENT IN THE 61^{O}C TRANSITION OF HUMAN ERYTHROCYTE MEMBRANE RELEVANT TO THE HEMOLYTIC ANEMIAS OF THE TYPE MEMBRANOPATHY

Iv. T. Ivanov [1*], A. Zheleva[2]

[1]Department of Physics, Biophysics, Roentgenology and Radiology, Medical Faculty, Trakia University, Stara Zagora, Bulgaria
[2]Department of Chemistry and Biochemistry, Medical Faculty, Trakia University, Stara Zagora, Bulgaria

ABSTRACT
Upon rapid (4^{o}C/min) heating of human erythrocytes and of NaCl-loaded erythrocyte membranes (EMs), suspended in low-salt/sucrose isotonic media, an intense out leakage of cytosolic ions is detected conductometrically at a temperature (T_g) that tends to 60.7^{o}C at zero heating rate. The T_g is considered as inducing for the hyperthermic (38-60^{o}C) activation of passive ion permeability and thermal hemolysis of erythrocytes. In erythrocytes and isolated EMs, pretreated (20-100 μM, pH 8.2) with 4,4'-diisothiocyano-2,2'-stilbene disulphonic acid, DIDS, powerful and specific inhibitor and thermal stabilizer of the anion exchanger, the EM band 3 protein (AE1), the T_g was increased by 3.5-4.5^{o}C. This result suggests that the anion exchanger was involved in the T_g transition of EMs. The W/S spectral parameter of EPR spin probe 3-Maleimido-PROXYL, bound at pH 6.9 to isolated EMs, decreased over the denaturation intervals of spectrin (48-52^{o}C) and of anion exchanger (63-72^{o}C), but increased within the interval (58-63^{o}C) of T_g transition. In line with previous reports, the latter result is interpreted in terms of a predenaturation transition in the anion exchanger. As this protein is changed in some hereditary anemic conditions the obtained results could be used to discriminate between membranopathy and other types of anemia.

Key words: erythrocyte membrane; anemia, membranopathy, band 3 protein; barrier function

INTRODUCTION

Hemolytic disorders and anemia occur when the erythrocyte survival time is less than 120 days and 15 days, respectively.Usually, the hemolytic disorder is compensated by increased erythrocyte production. The uncompensated hemolytic condition, anemia, leads to intravascular hemolysis and rise in CO content and could be directly detected by measuring the erythrocyte life time and unconjugated bilirubin, reticulocyte count, bone marrow aspirate, periperal blood smear, and decreased haptoglobin. The primary (intracorpuscular) causes of hemolytic anemia include hereditary membranopathy, hemoglobinopathy and enzymopathy of erythrocytes. Hereditary membranopathy, for example spherocytosis, is due to defects in spectrin, ankyrin and band 3 proteins resulting in disarranged protein interactions in the erythrocyte cytoskeleton and membrane.

There are much more causes for the secondary (extracorpuscular) anemia. Because the above enlisted methods are not specific it is frequently a problem to distinguish between both the primary and secondary anemia, as well as between the different types of primary anemia. The recently reported method of thermal analysis of the erythrocyte suspension impedance is sensitive to hereditary changes in major EM proteins and, hence, could help identify the primary membranopathy (1).

There are three major proteins of human EMs, namely, the peripheral protein spectrin, and the integral proteins glycophorin and the anion exchanger, AE1, the band 3 protein (2). Band 3

***Correspondence to:** *Ivan Tanev: Dept. Physics, Biophysics, Rentgenology and Radiology, Medical Faculty, Thracian University, Armeyska Str. 11, Stara Zagora 6000, Bulgaria. E-mail: ivanov_it@gbg.bg*

protein has a key position in the maintenance of erythrocyte structure and function. In senescent erythrocytes it changes generating an epitope on the cell surface leading to autologous IgG binding and consequent phagocytosis of cells (3). The scanning microcalorimetry of isolated erythrocyte membranes has demonstrated the thermal denaturations of spectrin at T_d = 49.5°C (A peak) (4) and of the transmembrane domain of the band 3 protein at T_d = 67°C (C peak) (5). The denaturation temperature of glycophorin (over 100°C) was much higher and of no medical relevance (6).

The above mentioned method consists in transient heating suspension of erythrocytes or isolated erythrocyte membranes and registering the changes in passive electric properties that depend on the major membrane proteins. Two prominent changes were detected at 50°C and about 61°C, respectively, considering the heating rate tend to zero. The first change undoubtfully implicates the denaturation of spectrin (4, 7), while the predenaturational transition of still unknown integral protein is involved in the second change. To help the applicability of the method in discriminating membranopathy this obscurity about the 61°C change should be eradicated. Indirect data indicating the possible participation of the anion exchanger in latter change have been reported (8). The presented study provides additional and convincing evidence in support of this case.

MATERIALS AND METHODS

Materials

4,4'-diisothiocyano-2,2'-stilbene disulphonic acid (DIDS), maleimido-PROXYL, dimethylsulfoxide (DMSO), sucrose and NaCl were purchased from Sigma chemical Co, St. Louis, MO, USA.

Isolation of erythrocytes and membrane preparation

Freshly drawn, heparinised, human blood samples were obtained from healthy volunteers and used in the same day. Erythrocytes were isolated by centrifugation and washed thrice in phosphate buffered physiological NaCl saline, 5PBS, pH 7.4. EMs were prepared diluting 1 volume of packed erythrocytes (intact and DIDS-treated) into 30 volumes of 4°C-cold hypotonic solution (1mM $MgCl_2$, 5 mM phosphate buffer, pH 8.0). The membranes were isolated by centrifugation at 15 000 x g and washed thrice in the same medium to pink colour. Prior to the last washing the isotonicity was restored to 145 mM NaCl, 1mM $MgCl_2$, 5 mM phosphate buffer, pH 7.4 and the membranes were resealed at 37°C for 15 min.

DIDS treatment of erythrocytes and membranes (9).

Washed erythrocytes or isolated EMs were suspended at a hematocrit of 0.05 in buffered 150 mM NaCl saline, pH 8.2, and treated with the indicated concentration of DIDS at dark and room temperature for 20 min. Prior to usage the cells (membranes) were three times washed of unbound DIDS with excess volume of 5PBS, pH7.4.

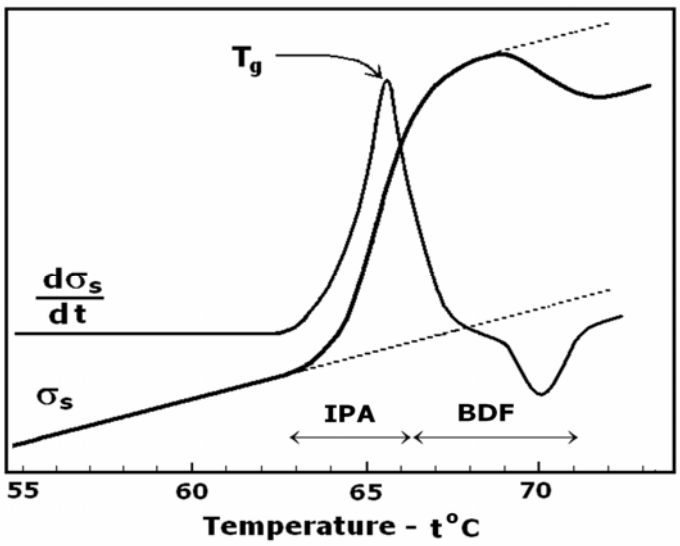

Figure 1. Temperature profiles of conductivity, σ_s, and of the temperature-derivative of conductivity, $d\sigma_s/dt$, for erythrocyte suspension. The suspension medium was isotonic solution of 50 mM NaCl and 200 mOsm sucrose. Hematocrit, frequency and the heating rate were 0.40, 20 kHz and 4 °C/min, respectively. IPA – temperature interval for ion permeability activation, BDF – temperature interval for barrier defects formation.

Conductivity data acquisition and processing

The derivative thermal analysis of suspension conductivity was carried out using SOLARTON SI 1260 Impedance-Gain Phase Analyser, England, as previously described (1n).

Spin Labeling and EPR study of isolated EMs

Proxyl - maleimide was freshly prepared as 50 mM stock solution in ethanol. Freshly isolated EMs were spin labelled at 37°C for 2 h in 5PBS, pH 6.9, hematocrit 0.1, that contained 0.15 mM Proxyl - maleimide. At this pH the spin labels primarily bound to SH groups on the protein surface (weakly immobilized, W-sites) as well as to internal SH-groups (strongly immobilized, S-sites). The amplitude ratio W/S of the EPR signals was used to monitor the influence of temperature on the conformation of EMs proteins **(Figure 3**). The spin labelled membranes were isolated and 4 times washed in excess volume of 5PBS. For the EPR investigation, packed spin labeled EMs were drawn into 50 μL glass capillaries (VWR, West Chester, PA) and sealed. EPR spectra were measured by X-band (9.844 GHz) Bruker BioSpin spectrometer (Germany) at room (23°C) temperature. The spectral parameters were as follows: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G; central field, 3510 G; sweep width, 100 G; resolution 1024 points; conversion, 300 ms; time constant, 1310.72 ms; sweep time, 307.2 s.

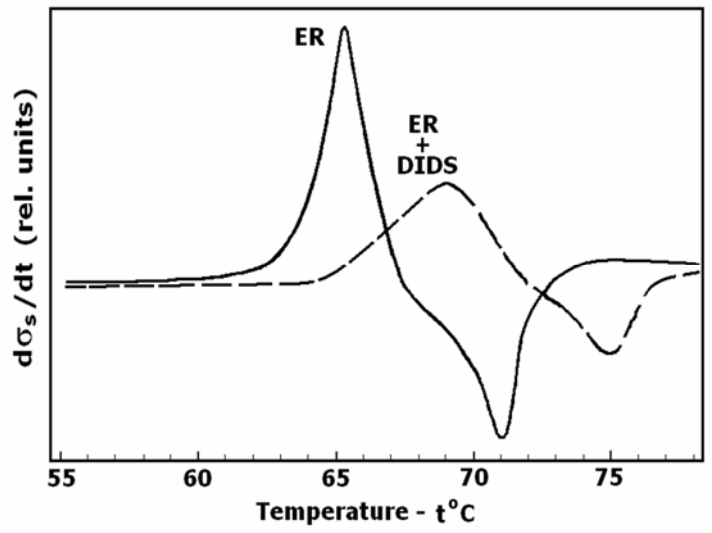

Figure 2. Effect of DIDS on the derivative conductivity thermogram of erythrocyte suspension. ER – intact erythrocytes; ER+DIDS – erythrocytes inhibited with DIDS (20 μM). The other details as for Figure 1.

RESULTS

The temperature profiles of the low-frequency (20 kHz) suspension conductivity, σ_s, and of its temperature-derivative, $d\sigma_s/d\theta$ over the 55-75°C range **(Figure 1)** both demonstrate a transition in EMs at the T_g temperature. The same types of curves were obtained heating whole human erythrocytes or NaCl - loaded isolated erythrocyte membranes, suspended in low-salt media. The positive peak at T_g on the derivative conductivity thermogram, coupled to the sigmoidal increase in σ_s over the 62-67°C interval, both are due to the downhill leakage of cytosolic ions and osmotically obliged shrinkage following the thermally-induced activation of EM ion permeability (10; 11). The T_g peak temperature tends to 61°C (T_{go}) when the heating rate tends to zero (not shown). The T_{go} is close to the temperature threshold of hemolysis (61-62°C (12)) and considerably beneath the denaturation temperature of the anion exchanger (67-68°C) (5). The peak at 50°C is out of the scope of this study and is not displayed on thermogram.

Stilbene sulphonic acid derivative DIDS is specific and powerful inhibitor and thermal stabilizer of the transmembrane domain of band 3 protein, increasing its denaturation temperature by far (13°C) (5). Similar to the microcalorimetric data for the calorimetric C peak (5) the DIDS apparently increased,

although to a lesser extent, the T_g peak temperature on the conductivity thermogram of whole erythrocytes **(Figure 2)**. Similar results were obtained with EMs isolated from DIDS-treated erythrocytes (not shown). DIDS significantly increased the half-width of G peak and shifted the temperature intervals of ion permeability activation and barrier defects formation by 4-5^{o}C **(Figure 2)**. The effect of DIDS was irreversible and did not change with decreasing the DIDS concentration from 200 μM to about 20 μM. The effect produced by DIDS on the EM transition at T_g temperature points out the anion exchanger as a possible participant in the hyperthermic modification of the barrier function during this transition and over the interval of hyperthermic (38-56^{o}C) temperatures.

Proxyl maleimide, a hydrophobic maleimide derivative, readily binds to the proteins of isolated human EMs, primarily to the major proteins of the anion exchanger and spectrin (13; 14; 15). On the other hand spectrin and glycophorin, that comprise almost 55% of EM protein content, could be readily discriminated based on the differences between their denaturation temperatures (49.5^{o}C and over 100^{o}C, correspondingly) and the T_g. Hence, the anion exchanger that comprised almost 80 % of the remainder of EM protein content could be associated with the unusual changes in the EPR spectral data about T_g.

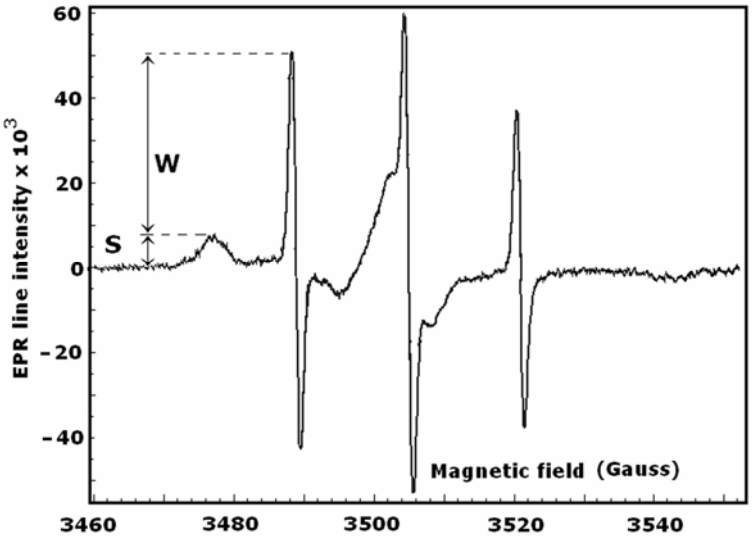

Figure 3. EPR spectra of EMs (10 mg protein/ml) spin labeled with 3-maleimide PROXYL (0.15 mM) for 2 h at 37^{o}C.

The EPR spectra **(Figure 3)** contained peaks corresponding to the number of strongly immobilized (slowly rotating, S) and weekly immobilized (fast rotating, W) probes covalently bound to SH-groups of major membrane proteins, primarily to spectrin and the anion exchanger. W/S ratio is the most commonly used spectral parameter particularly sensitive to the conformational changes in spin labeled proteins (16). For maleimide spin-labeled EMs the W/S ratio increases with temperature between 10 – 50^{o}C indicating that temperature affects the structure of membrane proteins intensifying the segmental motions of spin-labeled sites (17; 18; 19). In addition, **Figure 4** shows that the W/S ratio was increased at the temperature intervals where major membrane proteins denatured; 45-52^{o}C for spectrin unfolding and 64-72^{o}C for the denaturation of the anion exchanger. W/S decreased and S increased, however, over the temperature interval (58-63^{o}C) of the T_g permeability transition. These data indicated a decrease in the molecular motions close to the spin-labeled sites, primarily those bound to the anion exchanger.

Treatment of EMs with DIDS, prior to or after the spin labeling, resulted in about 15-fold reduction in the EPR spectral line intensity and elimination of the S line from the EPR spectrum (not shown). This possibly demonstrated that when intact EMs were treated with the spin label, most of the spin

labels actually bound the anion exchanger and reported changes in this protein.

CONCLUSSION

The results obtained with DIDS and 3-Maleimdo-PROXYL spin probe, bound to heated EMs indicate the implication of band 3 protein in the T_g transition in EMs that activate its ion permeability at hyperthermia. As the band 3 protein is changed in some hereditary membranopathic anemic conditions this result could be used to study and detect these types of anemia.

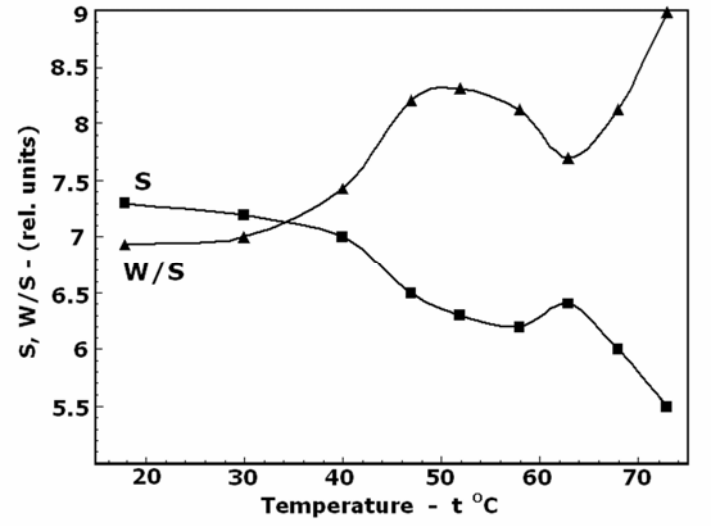

Figure 4. Temperature dependence of the S and W/S parameters of the EPR spectra of human erythrocyte membranes spin labeled with 3-maleimide PROXYL . For details see Figure 3.

REFERENCES

1. Ivanov, I. T., Tolekova, A., Chakaarova, P. Erythrocyte membrane defects in hemolytic anemias found through derivative thermal analysis of electric impedance. J. Biochem. Biophys. Methods, 70: 641-648, 2007.
2. Pasini, E. M., Kirkegaard, M., Mortensen, P., Lutz, H.U., Thomas, A.W., Mann, M. In-depth analysis of the membrane and cytosolic proteome of red blood cells, *Blood*, 108: 791-801, 2006.
3. Bosman, G. J., Were, J. M., Willekens, F. L., Novotný, V. M. Erythrocyte ageing in vivo and in vitro: structural aspects and implications for transfusion, Transfus. Med. 18: 335 – 347, 2008.
4. Brandts, J. F., Erickson, L., Lysko, K., Schwartz, A. T., Taverna, R. D. Calorimetric studies of the structural transitions of the human erythrocyte membrane. The involvement of spectrin in the A transition, Biochemistry. 16: 3450-3454, 1977.
5. Snow, J. W., Brandts, J. F., Low, P. S. The effects of anion transport inhibitors on the structural transitions in erythrocyte membranes, Biochim. Biophys. Acta. 512: 579-591, 1978.
6. Marton, L. S., Garvin, L. E. Subunit structure of the major human erythrocytes glycoprotein: depolymerization by heating ghosts with sodium dodecyl sulphate, Biochem. Biophys. Res. Commun. 52: 1457-1462, 1973.
7. Ivanov, I.T. Involvement of Erythrocyte Membrane Proteins in Temperature-Induced Disturbances of the Permeability Barrier, Membr. Cell Biol. 11: 45-56, 1997.
8. Ivanov, I. T., Brähler, M., Georgieva, R., Bäumler, H. Role of membrane proteins in thermal damage and necrosis of red blood cells, Thermochim. Acta. 456: 7–12, 2007.
9. Cabantchik, Z. I., Greger, R. Chemical probes for anion transporters of mammalian cell membranes. Am. J. Physiol. 262: C803–C827, 1992.
10. Ivanov, I. T., Benov, L. C. Thermohemolysis of human erythrocytes in isotonic NaCl / sucrose media during transient heating, J. Therm. Biology. 17: 381-389, 1992.
11. Ivanov, I. T. Allometric dependence of the life span of mammal erythrocytes on

thermal stability and sphingomyelin content of plasma membranes. Comp. Biochem. Physiol. Part A. 147: 876–884, 2007.
12. Coakley, W. T., Bater, A. J., Crum, L. A., Deeley, J. O. T. Morphological changes, haemolysis and microvesiculation of heated human erythrocytes, J. Therm. Biol. 4: 85-95, 1979.
13. Wyse, J. W., Butterfield, D. A. Electron spin resonance and biochemical studies of the interaction of the polyamine, spermine, with the skeletal network of proteins in human erythrocyte membranes. *Biochim Biophys Acta.* 941: 141–149, 1988.
14. Kłopocka, J. Flow cytometric analysis of eosin-5-maleimide binding to protein of band 3 and Rh-related proteins in diagnosis of hereditary spherocytosis, New Medicine, 1: 13-15, 2008.
15. Taylor, A. M., Zhu, Q. S., Casey, J. R. Cysteine-directed cross-linking localizes regions of the human erythrocyte anion-exchange protein (AE1) relative to the dimeric interface, Biochem. J. 359: 661-668, 2001.
16. Berliner, L. J. The spin-labeled approach to labeling membrane protein sulfhydryl groups, Ann. NY Acad. Sci. 414: 153-161, 1983.
17. Jozwiak, Z., Watała, C. Changes induced by chlorpromazine and heat in erythrocyte membranes, J. Therm. biol. 18: 83-89, 1993.
18. Szwarocka, A., Robak, T., Krykowski, E., Jozwiak, Z. Interaction of anthracyclines with human erythrocytes at hyperthermic temperature, Int. J. Pharmaceutics. 135: 167-176, 1996.
19. Wang, Y. Y., Li, X. J., Xin, W. J. Changes of the human erythrocyte membrane protein SH-binding site property with exposure to fluoride and three strong mutagens, Fluoride. 28: 193-200, 1995.