BAND 3 PROTEIN INVOLVEMENT IN THE 61°C TRANSITION OF HUMAN ERYTHROCYTE MEMBRANE RELEVANT TO THE HEMOLYTIC ANEMIAS OF THE TYPE MEMBRANOPATHY

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ABSTRACT
Upon rapid (4°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 (Tg) that tends to 60.7°C at zero heating rate. The Tg is considered as inducing for the hyperthermic (38-60°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'-diisothiocyanato-2,2'-stilbene disulphonic acid, DIDS, powerful and specific inhibitor and thermal stabilizer of the anion exchanger, the EM band 3 protein (AE1), the Tg was increased by 3.5-4.5°C. This result suggests that the anion exchanger was involved in the Tg 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°C) and of anion exchanger (63-72°C), but increased within the interval (58-63°C) of Tg 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...
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 $T_d = 49.5^\circ C$ (A peak) (4) and of the transmembrane domain of the band 3 protein at $T_d = 67^\circ C$ (C peak) (5). The denaturation temperature of glycoporin (over 100$^\circ C$) 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$^\circ C$ and about 61$^\circ C$, respectively, considering the heating rate tend to zero. The first change undoubtedly implicates the denaturation of spectrin (4, 7), while the pre-denaturational 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$^\circ C$ 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$^\circ C$ 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, $\sigma_s$, 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 $^\circ C$/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.

RESULTS
The temperature profiles of the low-frequency (20 kHz) suspension conductivity, $\sigma_s$, 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 $\sigma_s$ 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°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°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°C and over 100°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 $T_g$.

![EPR spectra of EMs (10 mg protein/ml) spin labeled with 3-maleimide PROXYL (0.15 mM) for 2 h at 37°C.](image)

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°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°C for spectrin unfolding and 64-72°C for the denaturation of the anion exchanger. W/S decreased and S increased, however, over the temperature interval (58-63°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.

CONCLUSION
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.

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