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Anion exchanger and the resistance against thermal haemolysis

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Abstract

4,4′-Diiso-thiocyanato stilbene-2,2′-disulphonic acid (DIDS) is a membrane-impermeable, highly specific covalent inhibitor and powerful thermal stabiliser of the anion exchanger (AE1), the major integral protein of erythrocyte membrane (EM). Suspensions of control and DIDS-treated (15 μM, pH 8.2) human erythrocytes were heated from $20°$ to $70°C$ using various but constant heating rates ($1–8°C/min$). The cellular electrolyte leakage exhibited a sigmoidal response to temperature as detected by conductometry. The critical midpoint temperature of leakage, T_{mo}, extrapolated to low heating rate ($0.5°C/min$) was used as a measure for EM thermostability. T_{mo} was greater for DIDS-treated erythrocytes, $63.2° \pm 0.3°C$, than for intact erythrocytes, $60.7° \pm 0.2°C$. The time, $t_{1/2}$, for 50% haemolysis of erythrocytes, exposed to $53°C$ was used as a measure for the resistance of erythrocytes against thermal haemolysis. The $t_{1/2}$ was also greater for DIDS-treated erythrocytes, 63 ± 3 min, than for intact erythrocytes, 38 ± 2 min. The fluorescent label N-(3-pyrenyl)maleimide and EPR spin label 3-maleimido-proxyl, covalently bound to sulphydryl groups of major EM proteins, were used to monitor the changes in molecular motions during transient heating. Both labels reported an intensification of the motional dynamics at the denaturation temperatures of spectrin ($50°C$) and AE1 ($67°C$), and, surprisingly, immobilisation of a major EM protein, presumably the AE1, at T_{mo}. The above results are interpreted in favour of the possible involvement of a predenaturational rearrangement of AE1 copies in the EM thermostability and the resistance against thermal haemolysis.

Keywords: *Erythrocyte membrane stability, thermal haemolysis, band 3 dimer, DIDS, heat injury*

Introduction

Thermally induced haemolysis of human erythrocytes has the basic prerequisites of thermal necrosis of cells including prelytic increase in ion permeability, swelling, and lysis. Under other non-physiological conditions eryptosis, a different type of haemolysis, occurs that shares the basic features of apoptosis: cell shrinkage, membrane blebbing, activation of proteases, and phosphatidylserine exposure at the outer membrane leaflet [1]. These observations substantiate the use of erythrocytes in model studies of hyperthermic effects on cells.

Upon rapid heating human erythrocytes do not lyse below $61–62°C$, the so-called temperature threshold of haemolysis (T_m). Above this temperature the rate of haemolysis sharply increases [2].

Within the interval between $45–56°C$, haemolysis has been found thermally activated with activation energy, E_a, 300 ± 20 kJ/mol [3–6]. Based on this high E_a many investigators [4–6] suggested that the prime target of haemolysis involves the thermal denaturation of a single protein or a group of similar proteins, but not melting of lipids. For human erythrocytes most expected participants in the prime target are their major proteins: the cytosolic haemoglobin [7], the peripheral protein spectrin [8] and the integral band 3 protein, the anion exchanger AE1 [9], while glycophorin is excluded because of its enormous thermal stability [10]. Thermal denaturations of major proteins appear as separate peaks on the differential scanning calorimetry (DSC) thermogram of isolated erythrocyte membranes (EMs).

At low heating rate (0.5°C/min) the denaturation of spectrin occurs at 49.5°C (A peak) [11], while the cytoplasmic domain of the band 3 protein denatures at 62°C (B_2 peak), and the membrane domain of the band 3 protein, MDB3, at $T_d = 67$°C (C peak) [12]. The denaturation temperature of haemoglobin, however, is higher (72°C).

More specific studies [6, 13–16] have presented evidence rejecting the participation of the thermal denaturations of haemoglobin and spectrin in the prime target of thermal haemolysis.

The participation of the band 3 protein, however, still remains obscure. Comparing the effect of heat on erythrocytes, haemosomes and liposomes, Lepock et al. [6] came to the conclusion that heat denaturation of the anion exchanger is not implicated in thermal haemolysis. By contrast, using specific modifications of the anion exchanger with DIDS and photochemical reagents other authors [9] suggested that the heat denaturation of the anion exchanger was involved in the thermal haemolysis and in the preceding ion permeability activation. A third group of investigators [17] claimed that the denaturation of the tertiary structure of EM proteins was not needed to induce the ion leakage from erythrocytes at T_m, however, a predenaturational transition in the anion exchanger was involved in the activation of ion permeability at T_m. This ambiguity is further deepened by the fact that band 3 protein occurs in dimeric and polymeric forms and, hence, it is not clear what kind of denaturation or predenaturational transition in latter protein might be involved in ion leakage and thermal haemolysis.

The participation of the band 3 protein in thermal haemolysis is important as this protein has a key position in the maintenance of erythrocyte structure and function. With about a million of copies per cell, it is the most abundant protein in EM. Moreover, it constitutes a subclass of the greater AE plasma membrane protein family responsible for the anion exchange diffusion and maintenance of intracellular pH and chloride content in most cells of vertebrate animals [18]. Anion exchange in many tissues shares (to varying degrees) with that in erythrocytes the sensitivity to the disulphonic stilbene class of inhibitors, electroneutrality of transport, and ion substrate specificities. Proteins, similar to the anion exchanger, were found in the membranes of bacteria and plant cells as well.

A number of studies indicate that electrolyte leakage precedes haemolysis and could be responsible for it [9, 13, 14, 19, 20]. Studies on whole [9, 13, 14] and reconstructed [13, 14] human erythrocytes, exposed to transient [13] and constant [9, 14] hyperthermic temperatures have clearly shown that thermal haemolysis has colloid-osmotic nature at its earliest stage. The rise in ion permeability that underlies haemolysis was studied over various temperature intervals: 47–65°C [19], 46–54°C [9], 50–58°C [14], and 38–57°C [21]. All studies indicated that the ion permeability obeys the straight line dependence in the Arrhenius plot with the same E_a of about 250 kJ/mol, indicating a single mechanism of activation over the whole 38–60°C range. Subsequently, both the electrolyte leakage and thermal haemolysis were related to a common thermally induced rearrangement of EM proteins, centred at T_m (61°C) [13, 14].

The investigations presented in this work were aimed at elucidating the possible involvement of the anion exchanger in the thermal stability of EM, as expressed by T_m, and resistance of erythrocytes against thermal haemolysis, $t_{1/2}$. In general, the thermal stability of proteins and cells is increased by a few number of agents including polyhydric alcohols and sugars [22], osmolytes [23], DNA and amino acids [24], some bivalent cations [25], heavy water and mild pressure [26], and some non-steroidal anti-inflammatory drugs and substances [27]. However, they all have a pervasive type of action and could not discriminate any particular cell protein as important for the values of T_m and $t_{1/2}$. DIDS is a membrane impermeable, covalent amino reagent. At low concentrations ($<30\,\mu$M) DIDS binds specifically to the anion exchanger of EM producing irreversible inhibition and thermal stabilisation of this protein [12, 17, 28, 29]. With the above in mind, we treated human erythrocytes with DIDS and verified the effect on their T_m and $t_{1/2}$. The results indicated that a rearrangement of band 3 protein, different from just thermal unfolding of its tertiary structure, was involved in electrolyte leakage and haemolysis at acute hyperthermia.

Materials and methods

Materials

DIDS, 3-maleimido-proxyl, dimethyl sulphoxide (DMSO), heavy water (D_2O), sorbitol, sucrose, raffinose, and NaCl were all purchased from Sigma, St. Louis, MO. N-(3-pyrenyl)maleimide was purchased from Fluka (Buchs, Switzerland). DIDS and dipyridamole was used as a stock solution in dimethyl sulphoxide.

Isolation of erythrocytes and preparation of erythrocyte ghost membranes

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 physiological NaCl saline. Special care was taken to remove the buffy

coat containing platelets and white blood cells as they are a source of unwanted proteolytic activity. Isolated erythrocyte ghost membranes were prepared diluting 1 volume of packed erythrocytes (intact and DIDS-treated) into 30 volumes of 4°C-cold hypotonic solution (1 mM $MgCl_2$, 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 until the membranes turned pink. Prior to the last washing the isotonicity was restored to the final concentration of 145 mM NaCl, 1 mM $MgCl_2$, 5 mM phosphate buffer, pH 7.4, and the membranes were resealed at 37°C for 15 min and isolated.

DIDS treatment of erythrocytes and erythrocyte ghost membranes

This protocol was carried out as previously described [29] with minor modifications to achieve optimal conditions. Aliquots of 0.15 mL packed erythrocytes or isolated erythrocyte ghost membranes were incubated at 4°C for 10 min in 10 mL media containing 15 μM DIDS, 10 mM Tris buffer, pH 8.2, 100 mM NaCl saline, and 2 mM EDTA and 100 mM sucrose as protectors. Control cells were prepared in the same way, except the suspension media did not contain DIDS. Prior to usage, the treated cells and ghost membranes were washed three times in excess volume of NaCl saline and the inhibition of the anion exchanger was verified. It is known that the addition of erythrocytes to an isotonic 20 mM NaCl/sucrose medium results in rapid acidification of this medium due to the exchange of inner Cl^- with outer OH^- through the anion exchanger of erythrocyte membranes [30]. The inhibition of the anion exchanger was assessed by suspending DIDS-treated cells in this medium and recording the decrease in the pH on a chart. The acidification was slower in rate and reduced in amplitude in comparison with that produced with intact cells.

Determination of erythrocyte resistance against thermal haemolysis, $t_{1/2}$

Aliquots of 0.15 mL packed erythrocytes, control and DIDS-treated, were suspended in 5 mL of 150 mM NaCl-saline that contained 3 mM EDTA, and incubated at 53°C. To assay the time course of thermally induced egress of haemoglobin, 0.075 mL aliquots were periodically withdrawn, diluted to 1.1 mL of 150 mM NaCl saline and the optical density measured at 700 nm (OD_{700}) at 23°C. The resistance against thermal haemolysis is defined by the time, $t_{1/2}$, needed to lyse 50% of cells according to the decrease in OD_{700} [20, 31].

Determination of erythrocyte plasma membrane thermostability, T_m

This method uses electrolyte leakage from heated cells as a means to assess the cell membrane thermostability and cellular injury by heat [31, 32]. It was previously applied on plant cells [33], erythrocytes [13, 14], and bacteria [34]. In contrast to other investigators [33] who have measured electrolyte leakage after prolonged detention at different temperatures, we continuously detected electrolyte leakage from erythrocytes during rapid heating of erythrocyte suspension with constant heating rate. The ion leakage was detected by the increase in low frequency (10 kHz) suspension conductivity, σ_s. The latter directly depends on the ion concentration of suspension media and is insensitive to cytosolic concentrations of ions.

Electrolyte leakage from erythrocytes, subjected to continuous heating over the temperature range from 20° to 70°C, exhibited a sigmoidal response to temperature. The critical, midpoint temperature, T_m, is defined as a measure for plasma membrane thermostability and for the critical killing temperature. To determine the T_m temperature, erythrocytes were suspended in a low-salt isotonic media, heated, and the electrolyte leakage assessed measuring σ_s. In substantiation of the conductivity measurements, parallel data on the leakage of K^+ from cells were obtained and published earlier [13, 21]. Briefly, 30 μL of erythrocytes were suspended in 70 μL of isotonic 50 mM NaCl/sucrose medium. The suspension was placed in a thin glass tube (ϕ 3 mm and L 100 mm) that contained two electrodes of thin platinum wire distanced at 10 mm under alternating voltage of 100 mV, 10 kHz. The glass tube was tightly incorporated within an aluminium block, heated with a constant heating rate, $V = d\theta/dt$, where t is the time elapsed and θ is the block temperature, measured by a thermocouple. During the heating, the suspension conductivity, σ_s, was continuously measured and the output signal of the conductometer, U_s, together with the signal from the temperature sensor were transmitted into a personal computer.

Similar to the conductivity of each electrolyte solution, σ_s exhibits a Boltzman type of dependence on θ. In a narrow temperature interval this dependence is reduced to closely linear one: $\sigma_s = \sigma_{so}(1 + K_s\Delta\theta)$ [35], where K_s is the temperature coefficient of σ_s. It was not informative to this study and was circumvented using the temperature-derivative of σ_s that could be expressed as $\partial\sigma_s/\partial\theta = \sigma_{so}.K_s$ (Figure 1). During the heating, only K_s is allowed to vary. At low frequency (10 kHz) the volume fraction of cells and the extracellular concentration of ions are major determinants of σ_s, hence, σ_s directly depends on the out leakage of ions. At a

steady-rate heating, the thermogram of $\partial\sigma_s/\partial\theta$ appeared as a horizontal line, unless K_s changed causing a sharp peak around the inducing temperature, T_m (Figure 1). A Solarton 1260 impedance/gain-phase Analyser (Farnborough, UK) and VersaSTAT 3 F (Princeton, NJ) were used to measure σ_s in order to determine T_m with greater accuracy. The reproducibility of determining T_m in repeated experiments was within $\pm 0.2°C$.

Spectrofluorometric monitoring of the dynamics of EM major proteins using PyM excimer/monomer fluorescence ratio

Emission of the excimer (short for exited dimer) of fluorescent dye N-(3-pyrenyl)maleimide (PyM), bound to the major proteins of EM, is used to assess the intramolecular dynamics of these proteins. Upon increased intramolecular movement a new fluorescence band of PyM appears attributed to the emission of an excited dimer formed by the combination of an excited singlet dye molecule with another unexcited one. PyM was freshly prepared as 0.9 mg/mL stock solution in DMSO. A 5-µL solution of PyM was added to 3 mL of isolated EMs (0.3 mg protein/mL) suspended in deaerated 5PBS, pH 7.4, 20 min prior to the spectrofluorometric measurements. The final concentrations of DMSO and PyM were 0.17% (v/v) and 1.5 µg/mL, respectively. Fluorescence was excited at 350 nm and

detected at 402 nm with intensity I_m for monomeric and at 421 nm with intensity I_e for excimeric emission, respectively. The excimerisation coefficient of PyM was estimated by the ratio of I_e/I_m, which was an inverse measure of the dynamics of bound protein. The fluorescence measurements were carried out with a spectrofluorimeter Jobin Yvon, model JY3 D, equipped with a U10 thermostat (Medingen, Germany). The temperature of the measuring cuvette was controlled by Thermocouple Meter, Omega, Newport (Deckenpfronn, Germany), resolution 0.1°C. The cuvette was step-wise heated at the indicated temperatures for 4.5 min. Fluorescence spectra were corrected by the blank fluorescence in the absence of fluorescent label. Due to the strong temperature variations in the intensity of membrane fluorescence, different apparatus magnifications were used and calibrated to the reference fluorescence intensity of the PyM label, dissolved in ethanol and kept at room temperature.

Spin labelling and EPR study of EMs

A 50-mM stock of 3-maleimido-proxyl was freshly prepared in ethanol. EMs isolated from fresh human erythrocytes were spin-labelled at 37°C for 2 h in 5PBS (a solution of 145 mM NaCl and 5 mM phosphate buffer, pH 6.9) that contained 0.15 mM 3-maleimido-proxyl, haematocrit 0.1. At this pH value the spin label bound primarily to the SH groups of EM proteins. Amongst EM proteins the anion exchanger and spectrin were predominantly labelled as the third major protein, glycophorin, do not contain SH groups. The spin-labelled membranes were washed four times in excess volumes of 5PBS. For the EPR investigation, packed spin-labelled 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 (Bruker Biospin, Rheinstetten, Germany) at room temperature (23°C). The spectral parameters were as followed: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G (Gauss); central field, 3510 G; sweep width, 100 G; resolution 1024 points; conversion, 300 ms; time constant, 1310.72 ms; sweep time, 307.2 s.
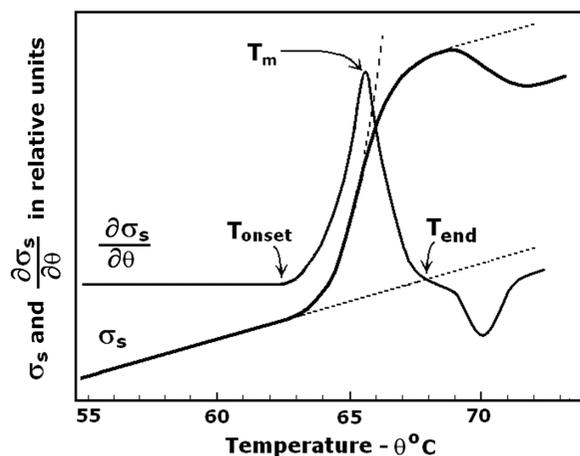

Figure 1. Electrolyte leakage from transiently heated erythrocytes as evidenced by the temperature dependence of suspension conductivity, σ_s, and of its temperature derivative, $\partial\sigma_s/\partial\theta$. The suspension medium was a low-salt isotonic solution of 50 mM NaCl and 200 mOsm sucrose. Haematocrit, frequency and the heating rate were 0.25, 10 kHz, and 4°C/min, respectively. Arrow indicates the mid-point inducing temperature, T_m, for the ion leakage at this heating rate. T_{onset} and T_{end} indicate the onset and end of the electrolyte leakage. T_m represents the thermal stability of erythrocyte membrane. Dotted line indicates the Boltzman-type temperature dependence of σs.

Results

Upon transient (4°C/min) heating of erythrocytes an abrupt leakage of cytosolic ions took place within the 62–67°C interval as detected by the increase in low frequency suspension conductivity, σ_s (Figure 1). The leakage exhibited a sigmoidal curve with mid-point temperature, T_m, at about $64.2° \pm 0.2°C$ at this heating rate. The same result was obtained with

resealed erythrocyte ghost membranes (not shown). The temperature derivative, $\partial\sigma_s/\partial\theta$, of the leakage was represented by a bell-shaped curve with top temperature centred at T_m. Because of its convenience, only this temperature derivative is shown in further experiments.

The value of T_m depended on heating rate and when the heating rate was extrapolated to low values (0.5°C/min), T_m tended to 60.7°C (T_{mo}) [21]. The T_{mo} was close to the temperature threshold of haemolysis, 61–62°C [2], and was considerably beneath the denaturation temperature (67–68°C) of MDB3 at low (0.5°C/min) heating rate [12].

To provide osmotic protection to cells during heating, the cells were suspended in isotonic, low-salt media, containing sucrose. Hence, the hyperthermia-induced ion leakage was enhanced, producing concomitant osmotic shrinkage of cells without haemolysis [13]. To study the role of the osmotic protector, sucrose was substituted by other osmotic protectors, sorbitol and raffinose. The molecular size of these protectors was ranked in the order sorbitol < sucrose < raffinose. No change in the ion leakage curve was evident using sorbitol, sucrose and raffinose (Figure 2). This finding indicated that the ion permeability of EM at T_m was only modified without producing barrier defects permeable for particles bigger than the inorganic cytosolic ions. The negative peak that followed the ion leakage peak indicated a threshold decrease in σ_s, inward diffusion of the osmotic protector and osmotic swelling of erythrocytes. This result demonstrated the appearance of barrier defects of a size that increased with the temperature above T_m. The negative peak was

less pronounced in the ghost membranes, compared to that in intact erythrocytes (not shown).

At micromolar (5–30 μM) concentrations, DIDS is a highly specific inhibitor and powerful stabiliser of the structure of MDB3, increasing its denaturation temperature by about 13°C [12, 17]. In this study the specific and covalent binding of DIDS (15 μM, pH 8.2) to the anion exchanger of erythrocytes irreversibly increased at a lesser, but still significant extent (2.5°C), the thermal stability of erythrocyte plasma membrane, T_m, in respect of control cells (Figure 3 and Table I). The DIDS treatment of erythrocytes was carried out at optimal conditions (see below). The same result was obtained with erythrocyte ghost membranes, isolated from DIDS-treated erythrocytes, and with intact ghost membranes,
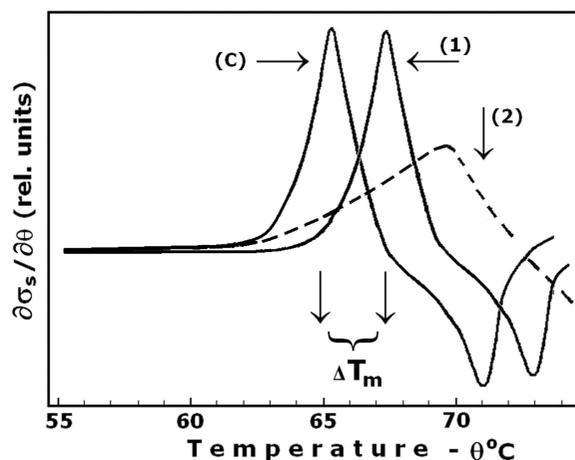
Figure 3.   Effect of DIDS on the ion leakage and thermal stability of erythrocyte membrane, T_m. The erythrocytes were treated at optimal conditions (100 mM NaCl, 10 mM Tris buffer, pH 8.2, 2 mM EDTA, and 100 mM sucrose, haematocrit 0.015, 4°C for 10 min) with 15 μM DIDS (curve 1) and without DIDS (curve C – control). The curve 2 represents irregular pattern of ion leakage due to DIDS binding at non-optimal conditions. The other details are as for Figure 1. Number of experiments, $N = 10$, a typical one is shown.
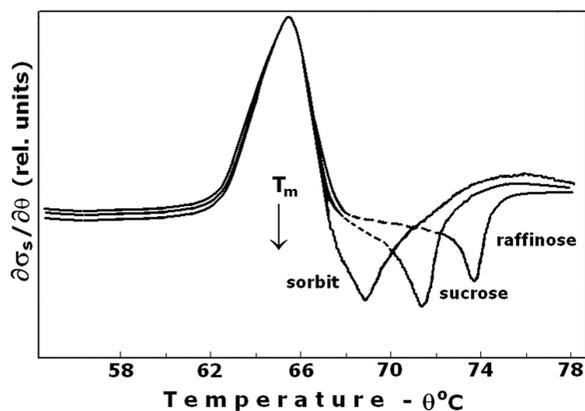

Figure 2.   Ion leakage at the presence of various osmotic protectors. The erythrocyte suspension medium was an isotonic solution that contained 50 mM NaCl and 200 mOsm of the applied osmolyte; sorbitol, sucrose and raffinose. Positive peak depicts the ion leakage, the consequent negative peak corresponds to the inward diffusion of the osmolyte. The other details are as for Figure 1.

Table I. Membrane thermostability and resistance against thermal haemolysis of control and DIDS-treated erythrocytes. The erythrocytes were treated at optimal conditions (100 mM NaCl, 10 mM Tris buffer, pH 8.2, 2 mM EDTA, and 100 mM sucrose, haematocrit 0.015, 4°C for 10 min). The thermal resistance is defined by the time, $t_{1/2}$, of exposure to 53°C resulting in 50% haemolysis. Membrane thermostability is defined by the temperature, T_m, that induces electrolyte leakage during heating of erythrocytes with low rate (0.5°C/min).

Cells under study	Thermal resistance, $t_{1/2}$ (min)	Membrane thermostability, T_m (°C)
Control erythrocytes	38 ± 2	60.7 ± 0.2
DIDS-treated erythrocytes	63 ± 3	63.2 ± 0.3

subsequently treated by DIDS (not shown). The temperature interval for formation of barrier defects in the plasma membrane of erythrocytes has also shifted to the higher temperatures (Figure 3). The effect of DIDS on ion leakage depended on the pH at which the DIDS treatment of cells was carried out. The maximum effect was at pH 8.2, whereat T_m increased by about 2.5°C, and the effect strongly decreased at pH 7.4 and pH 9.0 (not shown).

The covalent binding of DIDS to the anion exchanger of erythrocyte membranes, carried out at optimal conditions, also had an irreversible and positive impact on the resistance of erythrocytes against thermal haemolysis (Figure 4). The time, $t_{1/2}$, for 50% haemolysis of DIDS-treated cells, 65 min, was much larger than that of control cells, 38 min (Table I).

The DIDS treatment of erythrocytes was, however, accompanied by a less pronounced adverse effect, secondary to its beneficial impact on the stability of membranes (T_m) and cells ($t_{1/2}$). According to the data in Figure 4, thermal lysis of control cells commenced after a lag period during which all cells retained their haemoglobin. However, the lag period of DIDS-treated cells was absent and the haemolysis commenced immediately after their exposure to the haemolytic temperature. In other words, while DIDS treatment made the most of the cells more resistant to haemolysis, a little portion of cells, possibly the oldest one, became thermally sensitised. This effect of thermal sensitisation was not just due to the exposure of cells to the non-physiological, alkaline pH during DIDS treatment,
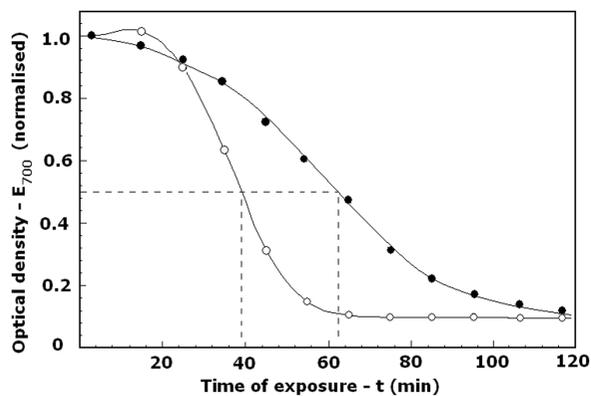

Figure 4. Effect of DIDS on the resistance of erythrocytes against thermal haemolysis, $t_{1/2}$. Cells treated with DIDS (•) or without DIDS (○) at optimal conditions (see Figure 3 for details) were exposed to 53°C for the indicated time intervals. The extent of haemolysis was determined by the optical density of the suspension at 700 nm (E700), normalized to its initial value. The initial and final values of E700 corresponded to 0% and 100% haemolysis, respectively. The $t_{1/2}$ indicated the time for 50% haemolysis. Number of experiments, $N = 4$, a typical one is shown.

as the effect persisted when the DIDS treatment was carried out at pH 7.8 and even pH 7.4 (not shown). In addition, the DIDS-treated erythrocytes displayed both increased resistance to haemolysis (Figure 4) and a regular pattern of ion leakage (Figure 3, curve 1) only when the DIDS treatment was conducted at properly designed optimal conditions. The latter conditions included low concentration of DIDS (less than 20 μM), cold (4°C), short exposure time (10 min), and the presence of EDTA (2 mM) and sucrose (100 mM). At other conditions the DIDS treatment resulted in cellular injury, evidenced by an irregular ion leakage pattern (Figure 3, curve 2) and lack of effect on the resistance against haemolysis (not shown). The nature of this secondary effect of DIDS, minimal at the optimal conditions for DIDS treatment, was not the subject of this study.

The deuterium oxide, D_2O, is a general thermal stabiliser of proteins and protector of cells against hyperthermia [36]. The haemolysis of human erythrocytes exposed at 55°C was protected by D_2O [37]. In this study, the substitution of 90% of the erythrocyte suspension water by D_2O increased T_m by $1.1° \pm 0.2°C$ (data not shown).

We further treated EMs with the highly lipophilic maleimide derivatives, 3-maleimido-proxyl and N-(3-pyrenyl)maleimide, at pH 6.9–7.5 whereat these reagents bind to protein SH groups, predominantly to those of the major EM proteins. Since glycophorin A does not contain SH groups [38] the predominant number of labels bound to the anion exchanger and spectrin [39–41]. Hence, these labels could report on changes in motional dynamics due to the denaturation of the anion exchanger and spectrin that could be conveniently discriminated, based on the difference in their denaturation temperatures, 67°C and 49.5°C, correspondingly. Figure 5 exhibits the temperature profiles of the excimerisation coefficient of N-(3-pyrenyl)maleimide, covalently bound to intact and DIDS-treated EMs. In general, the excimerisation of the label decreases when the motional dynamics of atoms, close to the binding sites of protein, increases. In the temperature interval (20–45°C) of no protein denaturation, the temperature dependence of the excimerisation is expressed by a slant curve indicating gradual temperature intensification of the movements within EMs. In addition to that general temperature dependence several threshold changes in excimerisation were exhibited around specific temperatures. The first one was the slight threshold reduction in the excimerisation within the interval of 48–52°C. It could be attributed to the denaturation of undermembrane spectrin network and related intensification of the motional dynamics of EM integral proteins, including that of AE1 [42]. A similar, although much stronger (5–8×) abrupt reduction in the
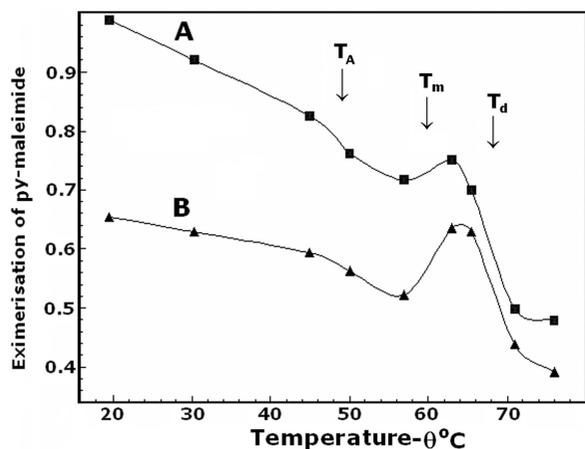
Figure 5. Effect of step-wise increase in the temperature on the excimerisation coefficient of N-(3-pyrenyl)maleimide bound to intact (A) and DIDS-treated isolated EM (B). Fluorescence was excited at 350 nm and detected at 402 nm with intensity I_m for monomeric and at 421 nm with intensity I_e for excimeric emission, respectively. Excimerisation coefficient was calculated as the ratio of I_e/I_m. The final concentrations of EM and N-(3-pyrenyl)-maleimide were 0.3 mg protein/mL and 1.5 µg/mL, respectively. The EMs were exposed for 4.5 min at each indicated temperature. Arrows indicate the inducing temperature for the ion leakage, T_m, the spectrin denaturation temperature, T_A [11], and the denaturation temperature, T_d [12, 17], of the MDB3 at low (0.5°C/min) heating rate.

excimerisation was detected over the temperature interval 64–70°C where the anion exchanger denatured. The excimerisation strongly increased within the intermediate temperature interval around T_m, including the 58–63°C interval for intact EMs and 58–66°C interval for DIDS-treated EMs. This indicated subsidence in the molecular motions close to the SH groups of some EM protein, presumably those of the anion exchanger.

Similar results were obtained with isolated EMs spin-labelled with 3-maleimido-proxyl. The EPR spectra (Figure 6A) contained two peaks with amplitudes corresponding to the number of strongly immobilised, slowly rotating (S amplitude) and weakly immobilised, fast rotating (W amplitude) spin labels, covalently bound to the SH groups of major EM proteins [43], predominantly those of spectrin and the anion exchanger [44–46]. The temperature dependence of the S amplitude (Figure 6B) just coincided with that of the excimerisation of N-(3-pyrenyl)maleimide (Figure 5). It indicated a decrease in the number of strongly immobilised sites within the denaturation intervals of spectrin and the anion exchanger and increase in this number around the T_m temperature. The temperature profile of the W/S ratio (Figure 6B) indicated similar changes in the molecular motions of EM major proteins.
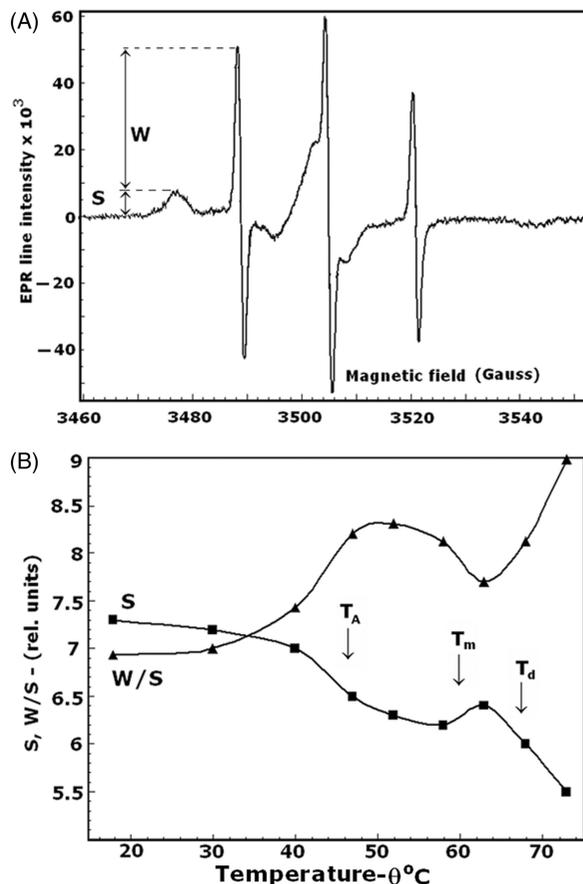

Figure 6. EPR spectrum of isolated EMs spin labeled with 3-maleimido-proxyl (panel A). S and W are the amplitudes of the EPR signals, corresponding to the number of strongly immobilised and weakly immobilised spin labels, respectively, covalently bound to the major EM proteins. The temperature dependences of the S and W/S spectral parameters are shown in panel B. Arrows indicate the temperatures as explained for Figure 5.

Discussion

We report here a considerable enhancement of the EM thermal stability and of resistance against thermal haemolysis of erythrocytes, both consequent to the selective and irreversible binding of DIDS to the EM anion exchanger. These findings led to the assumption that the anion exchanger was involved in the hyperthermic modification of ion permeability and haemolysis at hyperthermia. This assumption is consistent with the following reports on the molecular events that led to ion leakage and thermal haemolysis.

Several lines of evidence suggest that integral but not peripheral proteins participate in the EM rearrangement at T_m and in the resistance against thermal haemolysis [13, 47]. First, the resistance against thermal haemolysis of mammal erythrocytes, $t_{1/2}$, and thermal stability of their plasma membrane, T_m, have been found to display strong interspecies

differences which correlated to the naturally occurring variations in the sphingomyelin content of plasma membrane. The greater the ratio sphingomyelin/phosphatidylcholine, the greater were the T_m and $t_{1/2}$ [20, 21]. Second, the incorporation of sphingomyelin into human EMs has elevated T_m by $1° \pm 0.2°C$ and increased $t_{1/2}$ by 56%, while phosphatidylcholine produced practically no effect [31]. These findings are in accordance with the report that sphingomyelin, compared to phosphatidylcholine, confers more thermal stability to the integral proteins of EM and particularly to the MDB3 [48, 49]. Third, the EM rearrangement at T_m has demonstrated sensitivity to amphiphiles, similar to that of calorimetric C transition. For example, in contrast to B_2 and spectrin denaturation transitions, the calorimetric C transition was highly sensitive to various amphiphiles; local anaesthetics [50, 51], ethanol [52] and hydroxychloroaromatic compounds [53]. This is explained by the C transition implicating a change in the localized phospholipid region proximal to the band 3 protein, in addition to the MDB3 denaturation. Ethanol displayed just equal capacity to decrease T_m [13] and the denaturation temperature of MDB3 [52] which was 3.5 times greater than the capacity to decrease the denaturation temperature of spectrin. Hence, in the presence of 18% (v/v) ethanol, the T_m is lowered to 39°C while spectrin denatures at about 45°C [13]. Based on this outcome, interesting results have been obtained incubating erythrocytes at 39°C in the presence of 18% (v/v) ethanol for 3 min, as follows.

The above exposure irreversibly induced the EM rearrangement at T_m as well as the accompanying permeabilisation, at the same time retaining the undermembrane spectrin network intact [17]. These erythrocytes were called erythrocytes that sustained sparing permeabilisation in the presence of 18% ethanol, briefly, sparingly permeabilised erythrocytes. SDS-PAGE, thermal gel analysis and microcalorimetric thermogram of the ghost membranes, isolated from sparingly permeabilised erythrocytes, did not detect denaturation of the tertiary structure of membrane proteins [54, 55, 17]. The denaturation temperature of spectrin did not change, whereas that of the anion exchanger decreased by 2.5°C compared to that of intact membranes [17]. The specific inhibition of the EM anion exchanger with DIDS, prior to or after the sparing permeabilisation, increased the denaturation temperature by 9.5°C only, instead of 13°C for inhibited intact membranes [17]. In contrast to the intact membranes, treatment of sparingly permeabilised membranes with the bifunctional amino reagent 4,4′-difluoro-3,3′-dinitrophenylsulphone (FNPS) produced extensive cross-linking of membrane proteins [54]. At low concentrations of FNPS (10 µM)

the band 3 was the protein predominantly cross-linked, indicating reduction in the average distance between its copies, i.e. clusterisation. In accordance with the last finding, the spectrofluorometric studies of sparingly permeabilised membranes demonstrated a significant diminution in the lipid–protein contact zone according to the reduction of the energy transfer coefficient [55]. In conclusion, these data support the assumption that a thermally induced rearrangement of EM band 3 protein, different from the final unfolding, was involved in the T_m permeabilisation and thermal haemolysis.

There is a plethora of reports on the structure and function of the band 3 protein and its interaction with DIDS [56]. With a molecular weight of about 95 kDa the band 3 polypeptide forms two domains. The 40 kDa N-terminal domain is located within the cytoplasm and serves as a binding site for the undermembrane skeleton and other proteins, while the 55 kDa C-terminal domain (MDB3) is membrane-inserted and accomplishes an electroneutral exchange diffusion of anions.

The band 3 protein exists as a mixture of dimers and larger oligomers, the predominant (70%) species being a dimer [57, 58]. It could not be dissociated to monomers, other than by protein denaturation. The fraction of band 3 not associated with the cytoskeleton is almost entirely dimeric. The higher oligomers interact with the cytoskeleton, increase in amount with cell age, and are held together by interactions of the cytoplasmic domain. Interestingly, band 3 oligomeric state could be reversibly changed by altering the pH of the solution. There is one DIDS binding site per each band 3 polypeptide. The single bound DIDS molecule can react covalently with 2 different lysine residues [59]. Based on the rate of reactions, the primary site of binding is Lys-539 while Lys-851 appears as a secondary site of binding. It is not known whether these modalities were related to the positive and adverse effect of DIDS on the thermal stability of EM (Figure 3) and thermal resistance of erythrocytes (Figure 4).

In this work we studied a particular rearrangement of EMs at T_m (60.7°C) related to the ion leakage, EM protein immobilisation, and haemolysis due to high thermal dose and temperatures. The results presented led to the assumption that despite its considerably higher denaturation temperature (67°C) a large body of the band 3 protein copies was involved in this rearrangement. These results also led us to assume that the band 3 protein dimer was subject to a thermally induced alteration, different from the final unfolding and reduction to monomers. Most likely, this rearrangement included clusterisation of band 3 dimers into larger polymers with consequent immobilisation. This assumption is consistent with the spectrofluorometric and EPR

data (Figures 5 and 6) indicating decrease in the EM motional dynamics at T_m that could be associated, based on indirect data, with the anion exchanger, too. Melittin, zinc ions and acridine orange also cluster the band 3 dimers to tetramers leading to complete immobilisation of band 3 protein as detected by the dramatic decrease in the anisotropy of fluorescent labels bound to this protein [60]. Melittin, a key component of bee venom, is a powerful in vitro haemolytic factor, while zinc ions produce competitive and reversible inhibition of melittin-induced haemolysis [61]. In in vivo and in vitro senescent erythrocytes the conformation of MDB3 changes causing increased clusterisation of band 3 dimers [62, 63], which in turn leads to opsonisation and phagocytosis [64]. In conclusion, the obtained results possibly indicate the involvement of the anion exchanger in the EM rearrangement at T_m and suggest clusterisation of its dimers similar to what happens in other biological events and, because of the almost ubiquitous distribution of this protein, to other cell types as well.

Conclusion

The fluorescent label N-(3-pyrenyl)maleimide and the spin label 3-maleimido-proxyl, covalently bound to SH-groups of EM proteins, both revealed an abrupt increase in the molecular mobility at the denaturation temperatures of spectrin, 49.5°C, and of the anion exchanger, 68°C. In addition, these labels revealed an immobilisation of membrane protein, presumably the anion exchanger, at the intermediate T_m temperature (60.7° ± 0.2°C), corresponding to the heat injury, ion leakage and haemolysis of erythrocytes. Specific and covalent binding of DIDS to the anion exchanger of human erythrocytes, conducted at 15 μM, 4°C, 2 mM EDTA, 100 mM sucrose for 10 min, increased the thermal stability, T_m, of plasma membranes by about 2.5°C and the resistance against thermal haemolysis by 65%. These data provide new insight into the molecular mechanisms leading to ion leakage and haemolysis of erythrocytes resulting from high temperature and thermal dose.

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